(1)

FEB 1958
LIBRARY
POFI
FISH & WILDLIFE
SERVICE

JOURNAL

OF THE

TOKYO UNIVERSITY OF FISHERIES

VOL. 43, NO. 1.

CONTENTS

OGINO, C. and TAKI, Y.: Studies on the tannin of brown alga, *Sargassum ringgoldianum* HARV. 1– 5

OGINO, C.: An improved procedure for paperchromatgraphic determination of amino acids..... 7– 11

TANAKA, K. and TANAKA, T.: Effect of differences of initial and final temperature of whalemeat when it is taken into and out of freezer on drips..... 13– 17

TANAKA, K. and TANAKA, T.: Drip from frozen whalemeat affected by freezing rate and air temperature in air defrosting..... 19– 23

OSAKABE, I., FUKUSHIMA, K., OKADA, I. and HASEGAWA, M.: Polarographic studies on cuttle fish meat. Part I..... 25– 35

ONO, T., NAGAYAMA, F., OSAWA, H. and TAKAHASHI, B.: Enzymatic studies on the glycolysis of fish muscle I. Activity of phosphorylase 37– 47

ONO, T., NAGAYAMA, F. and SASAKI T.: Biochemical studies on the Vitamin A in fish viscera IV. Lipoxidase in liver and dark muscle 49– 57

HOSHINA, T.: Further observations on the causative bacteria of the epidemic disease like furunculosis of rainbow-trout..... 59– 66

HOSHINA, T. and SANO, T.: On a trypanosome of eel 67– 69

HOSHINA, T. and HOSODA, S.: On a new myxosporidian species, *Thelohanellus cyprini*, n. sp., parasitic in the fin of *Cyprinus carpio* 71– 73

SANO, T.: Haematological studies of the culture fishes in Japan. 1. On the blood of eel 75– 79

INABA, D., NOMURA, M. and NAKAMURA, M.: Preliminary report on the spawning of Grass-carp and Silver-carp in the Tone River, Japan and the development of their eggs 81– 96

TAKAGI, K.: Descriptions of some new gobioid fishes of Japan, with a proposition on the sensory line system as a taxonomic appliance 97–126

THE TOKYO UNIVERSITY OF FISHERIES
SHIBA KAIGANDŌRI 6, MINATO-KU, TOKYO, JAPAN

March, 1957

JOURNAL OF THE TOKYO UNIVERSITY OF FISHERIES

Editors for this Journal

Ikunosuke OKADA, Professor, Dr.

Hiroshi NIINO, Professor, Dr.

Itsuo KUBO, Professor, Dr.

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

STUDIES ON THE TANNIN OF BROWN ALGA, *SARGASSUM RINGGOLDIANUM* HARV.

Chinkichi OGINO and Yasuhiko TAKI

(Received Feb. 5, 1957)

The occurrence of tannin-like substance in several marine brown algae has been mentioned first by KYLIN[1]. Namely, he has observed that the contents of fucosan vesicles of some brown algae show the reaction of phlorogrucinol. He has named fucosan for the substance responsible. After death of algae, fucosan is converted by oxidation to a brown or black pigment, phycophaein. Little has been known, however, about the nature of fucosan as yet. TAKAHASHI[2] has isolated a black pigment, which is insoluble in water and upon being fused with KOH gives phloroglucinol, from an alcoholic extract of air-dried brown alga, *Ecklonia cava*. This pigment may be considered as an oxidation product of fucosan but no more detailed relationship between them has yet been clarified.

NOGUCHI[3] has reported the presence of tannin-like substance in *Sargassum Ringgoldianum* from the facts that an aqueous extract obtained at an elevated temperature has an ability of precipitating gelatin and a power of tanning as well. SUEHIRO and SEARASHI[4] have observed that the boiling water-extracts of a number of brown algae show the properties of tannin. From qualitative tests, they have supposed that the tannins of *Sargassum Ringgoldianum*, *S. Thunbergii*, and *Ecklonia Kurome* may belong to a catechol group. However, since the reactions of such crude extracts as above may have certainly been modified as a result of the lability of tannin itself, it will be dangerous to classify the tannin of algae simply from their reactions.

The occurrence of tannin or related substances in some brown algae may be easily recognized from the following facts that: 1) the aqueous extract of fresh brown algae shows violet or blue colour by addition of $FeCl_3$, 2) when certain brown algae are dried in the air, the colour of them turns to black, presumably due to oxidation of tannin or related substances, 3) the taste of some fresh brown algae is astringent. No detailed study, however, on the algal tannin has been made. The study of tannin or tannin-like substance of brown algae is considered to be important from the viewpoints of biochemistry and industrial utilization of algae. The purpose of the present investigation is to elucidate the nature of algal tannin.

In the present study, the tannin of considerable purity was isolated from an alcoholic extract of fresh brown alga, *Sargassum Ringgoldianum*, of which chemical and paperchromatographic properties were investigated.

The author wishes to express his gratitude to Dr. T. MIWA for his valuable advices rendered during the course of this work.

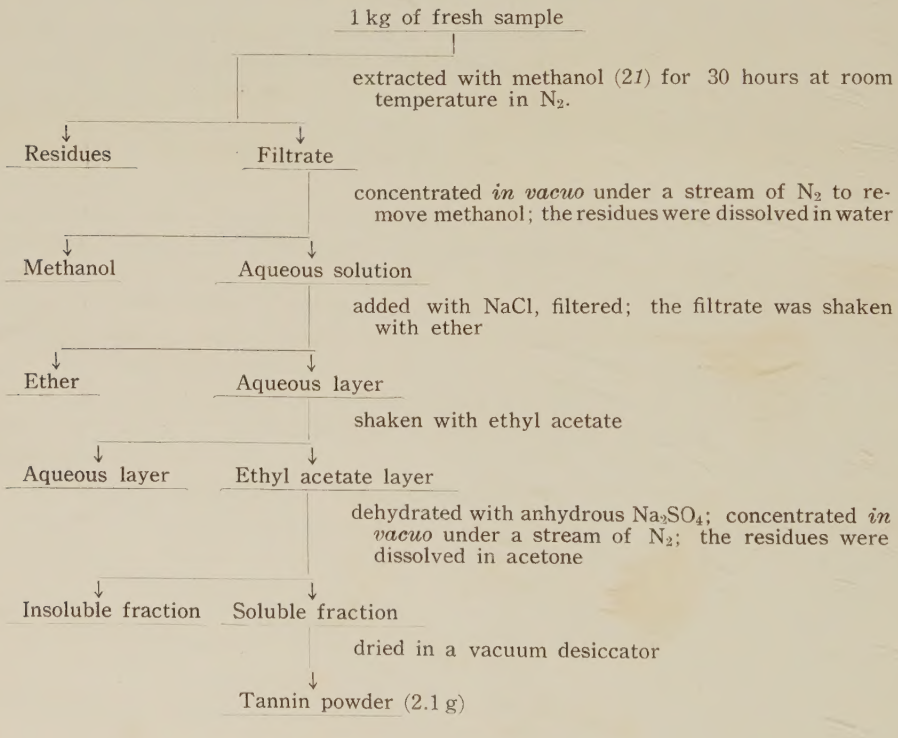
EXPERIMENTAL

1. Isolation of tannin from *Sargassum Ringgoldianum*.

Isolation of tannin was made according to a schema shown in Table 1. 1kg

of the fresh samples, collected Aug. 16, were cut into small pieces and once extracted with 2*l* of methyl alcohol at room temperature for 30 hours in a nitrogen atmosphere. The extract was concentrated in a reduced pressure under streaming nitrogen. The residues were dissolved in water and 15g of NaCl were added to it and filtered. The filtrate was shaken in a separating funnel with ether to remove substances soluble in ether. The aqueous layer was then shaken with ethyl acetate several times to dissolve tannin into the solvent. The combined solutions of ethyl acetate were dehydrated with anhydrous Na_2SO_4 and then evaporated to dryness in a reduced pressure under streaming nitrogen. The residues thus obtained were dissolved in acetone and filtered. The filtrate was transferred into a PETRI-dish and dried in a vacuum desiccator at room temperature. Thus, the tannin-powder, which was pale yellowish brown, was isolated from the sample with an yield of 2.1 g. The tannin could easily be torn out from the PETRI-dish. The purity of the isolated tannin analyzed by the hide powder method was 93%.

Table 1. Isolation of tannin.


1 kg of fresh sample
extracted with methanol (2l) for 30 hours at room temperature in N2.
Residues
Filtrate
concentrated in vacuo under a stream of N2 to remove methanol; the residues were dissolved in water
Methanol
Aqueous solution
added with NaCl, filtered; the filtrate was shaken with ether
Ether
Aqueous layer
shaken with ethyl acetate
Aqueous layer
Ethyl acetate layer
dehydrated with anhydrous Na2SO4; concentrated in vacuo under a stream of N2; the residues were dissolved in acetone
Insoluble fraction
Soluble fraction
dried in a vacuum desiccator
Tannin powder (2.1 g)

2. Chemical properties of the tannin.

The chemical properties of the tannin preparation are summarized in Table 2.

Table 2. Chemical properties of the tannin.

Reagents	Solubility, colour- or precipitation-reactions
H_2O	soluble
Acetone	〃
Ethyl acetate	〃
Pyridine	〃
Methyl alcohol	〃
Ethyl alcohol	〃
Amyl alcohol	〃
Chloroform	insoluble
Carbon tetrachloride	〃
Ether	〃
Mohr's salt	bluish violet
$FeCl_3$	reddish violet
NaCN	yellow
NaOH	dark green
Lead acetate	yellow precipitate
Lead acetate and acetic acid	pale green precipitate
Gelatin	white precipitate
$CuSO_4$	greenish yellow precipitate
Bromine water	precipitable but slightly
HCl and formalin (heating)	perfectly precipitated
Fehling's solution	reduced

The tannin isolated is lustrous powder in appearance, pale yellowish brown in colour, and astringent in taste. Its aqueous solution reacts acidic to litmus and reduces the Fehling's solution. The tannin shows a hygroscopic nature and in the air turns to black slowly. The tannin is soluble in water, alcohols, pyridine, acetone, and ethyl acetate and insoluble in ether, chloroform, and carbon tetrachloride. The dilute aqueous solution of the tannin is coloured bluish violet by Mohr's salt and reddish violet by ferric chloride. The tannin is precipitated by geratin, lead acetate, and bromine water, and completely by heating with formalin and HCl.

3. Paperchromatography of the tannin.

The paperchromatographic behavior of the tannin was investigated by the method of OSHIMA *et al*[5]. A strip of filter paper used (Toyo, No. 50) was washed with dilute HCl and water before use. The solvent mixtures used were an upper layer of a mixture of *n*-butyl alcohol-acetic acid-1% $NaHSO_3$ (4:1:5) and phenol-1% $NaHSO_3$ (4:1). After development, the filter papers were sprayed with 0.5% $FeCl_3$ in methyl alcohol or 0.5% aqueous Mohr's salt to bring out all of the spots. The chromatograms thus obtained are shown in Fig. 1. The principal portion of the tannin did not travel with these solvent mixtures from a original line and a small portion of them was separated into 3 or 4 spots. By the two-

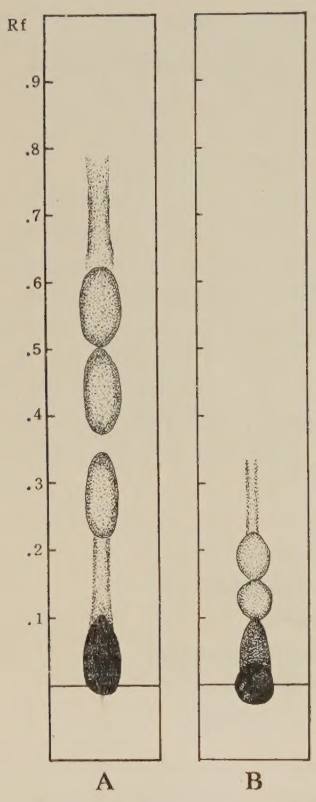


Fig. 1. Paperchromatograms of the isolated tannin.

Solvent mixtures: A. *n*-Butyl alcohol-Acetic acid-1% $NaHSO_3$ (4:1:5); B. Phenol-1% $NaHSO_3$ (4:1).

dimensional method, the tannin could not be separated on a paper sheet, perhaps due to oxidation of the tannin in drying of the solvent used in the first development.

4. Decomposition products by caustic alkali.

0.5g of the tannin were hydrolyzed by boiling them with 30% NaOH for 3 hours under a stream of nitrogen. The hydrolysate was acidified with H_2SO_4 and shaken several times with ether. The ether layers were combined together and then the ether was distilled off. The residues were dissolved in water. The aqueous solution obtained showed the reactions of phlorogrucinol; namely it gave a red stain on a pine shaving treated with concentrated HCl and violet colouration by phosphomolybdic acid and ammonia. A small portion of the sample solution and pure phlorogrucinol solution were applied to strips of filter paper and developed with the solvent mixtures of *n*-butyl alcohol-benzene-acetic acid-H_2O (1:5:1:0.5)[6] or *n*-butyl alcohol-acetic acid-H_2O (4:1:5). After development the chromatograms were sprayed with the dilute solution of diazobenzenesulfonic acid. The presence of phlorogrucinol in the sample was confirmed by the complete coincidence of Rf values of the spots of both sample and standard. Gallic acid was absent. The presence of other phenolic acids could not be demonstrated.

5. Hydrolysis by acid.

0.5 g of the sample were hydrolyzed by boiling with 10% H_2SO_4 for 27 hours in a current of nitrogen. During hydrolysis, a large amount of precipitates was formed. The hydrolysate was shaken with ether and after removal of ether, H_2SO_4 in the solution was precipitated with baryta, and filtered. To the filtrate lead acetate was added and filtered. The excess of lead in the filtrate was precipitated with H_2SO_4 and filtered again. The filtrate obtained neither reduced the Fehling's solution nor formed phenylosazone crystal of sugars. Thus, it may be said that the present tannin is not of a glycoside nature. The precipitates formed in hydrolysis did not give the Griessmyer's reaction, hence it is not correspondent to ellagic acid.

RESULTS AND DISCUSSION

The specimen isolated from *Sargassum Ringgoldianum* shows the properties of tannin in chemical features. From the properties described in the experimental section, the tannin can be said to belong to a catechol variety of condensed tannin according to the classification of FREUDENBERG. It contains phlorogrucinol nucleus, no sugars, and forms precipitate when hydrolyzed with acid.

By the paperchromatography, the tannin was separated to 3 or 4 spots besides a principal portion but it is not certain whether several kinds of tannin are existent as such in living alga or produced secondarily during treatment of the sample.

The tannin is oxidized readily in the air and turns to a black pigment. The blackning of some brown algae occurring when they are dried in the air may chiefly be due to oxidation of their tannins.

TAKAHASHI[2] has isolated a black pigment from air-dried brown alga, *Ecklonia cava*, which is insoluble in water and contains phlorogrucinol nucleus. The pigment may be oxidation product of tannin and may be identical with phycophaein. The present specimen is readily soluble in water and possesses every property of tannin of higher plants. The tannin can never be isolated from air-dried sample. The present specimen is presumably coincident with fucosan named by KYLIN[1], and hence it appears that fucosan is a sort of tannin or closely related compound to anthoxanthin pigment in its chemical character.

SUMMARY

The tannin has been isolated from fresh sample of brown alga, *Sargassum Ringgoldianum*, just collected from the sea, of which chemical and paperchromatographic analyses have been carried out.

The tannin likely inherent to this alga belongs to a catechol variety according to the classification of FREUDENBERG. The present specimen may be coincident with fucosan named by KYLIN[1].

REFERENCES

1) KYLIN, H.: *Z. physiol. Chem.*, **76**, 214 (1912).
2) TAKAHASHI, T.: *Tokyo Kogyo Shikenjo Hokoku*, **26**, 1 (1931).
3) NOGUCHI, E.: *Bull. Japan. Soc. Sci. Fish.*, **12**, 52 (1943).
4) SUEHIRO, Y. and SEARASHI, T.: *Bull. Physiograph. Sci. Research Inst. Tokyo Univ.*, No. 1, 44 (1948).
5) OSHIMA, Y., NAKABAYASHI, T., HADA, N. and MATSUYAMA, S.: *J. Agr. Chem. Soc. Japan*, **28**, 618 (1954).
6) NAKABAYASHI, T. and NISHIDA, S.: *J. Agr. Chem. Soc. Japan*, **26**, 333 (1952).

Digitized by the Internet Archive
in 2025

https://archive.org/details/journal-tokyo-university-fisheries_1957-03_43_1

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

AN IMPROVED PROCEDURE FOR PAPERCHROMATOGRAPHIC DETERMINATION OF AMINO ACIDS

Chinkichi OGINO

(Received Feb. 5, 1957)

Numerous investigations have reported on the determination of amino acids by paperchromatography. The paperchromatographic method for determination of amino acids is not time-consuming and not troublesome as compared with the microbiological or liquid chromatographic methods available, but in respect of the accuracy of estimation is generally lower than the latter.

In the determination of amino acids by paperchromatography, it is advisable that the development of amino acids is done by the one-dimensional method on a paper strip because of easiness in cutting out the spots and evading the decomposition of amino acids proceeding in a long time-development in the two-dimensional procedure. However, it is difficult in usual to obtain the spots of all amino acids as individual entities on paper by the one-dimensional method. McFARREN *et al*[1),2)]. have described the use of various buffered phenols and alcohols for development and estimation of 18 amino acids. The use of the buffered filter paper and solvent mixtures brings good results for obtaining compact and discrete spots. MARUTA *et al*.[3)] have employed the paper buffered at pH 5 with McILVAINE solution in their paperchromatographic determination of amino acids but their method is very complicated in its details.

In the present method, a mixture of amino acids were divided into monoamino acid- and diamino acid-fractions by treating them with ion exchange resin Amberlite IRC-50 buffered at pH 4.7 and subsequently the amino acids in each fraction were determined by the one-dimensional paperchromatography, using several buffered solvent mixtures and strips of filter paper. The method yielded highly reproducible results in determinations of amino acid content of protein.

This study was supported in part by a grant in aid for fundamental scientific research from the Ministry of Education to which the author offers his hearty thanks.

EXPERIMENTS AND RESULTS

1. Separation of amino acid mixture into monoamino acid- and diamino acid-fractions with ion exchange resin Amberlite IRC-50.

The separation of amino acid mixture into monoamino acid- and diamino acid-fractions was made by KUNIN's method[4)], using ion exchange resin IRC-50. The resin column (1×40 cm in Na type) was washed thoroughly with 1N HCl, water, and then with 0.5N NaOH and finally buffered at pH 4.7 by washing with acetate buffer. The elution of adsorbed diamino acids was made by 1N HCl. The recovery of amino acids by this method was estimated with the mixture of

amino acids of known concentrations and the result is given in Table 1. As shown in the table, the amino acids contained in a mixture were grouped quantitatively into two fractions by this method.

Table 1. Separation of amino acid mixture into monoamino acid- and diamino acid-fractions with resin IRC-50.

	Total N taken	Total N found	Total N calculated	Recovery
	mg	mg	mg	%
A mixture of aspartic acid, glutamic acid, alanine, and glycine.	26.02	16.47	16.40	100.4
A mixture of arginine, histidine, and lysine.		9.71	9.62	100.9

2. Paperchromatography of amino acids.

The solvent systems employed, the kinds of amino acids to be separated as individual entities, and other conditions in chromatography are summarized in Table 2. The chromatograms prepared under the above conditions are shown in Fig. 1. The resolution of diamino acids was made by means of the solvent mixture of ROLAND and GROSS[5]. The filter paper used was Toyo No. 50 of 2×60 cm in size in all runs. The development was done by the descending method. The excess of solvent, which was flowed out from the lower end of the filter paper during 24 hours-development, was allowed to be sucked in into another filter paper. When the development was made by *o*-cresol or *n*-butyl alcohol-benzyl alcohol (1:1) mixture, the filter paper was buffered by dipping it in the pH 5 McILVAINE buffer solution. In this case, the over-drying of the filter paper must be avoided especially in the development of phenylalanine, leucine, and isoleucine with benzyl alcohol-butyl alcohol mixture. A considerable wetness of the filter paper was necessary for a good separation of above three amino

Table 2. Solvent systems, kinds of amino acids to be separated, treatment of filter paper, and the time of development.

Solvent systems	Amino acids to be separated	Filter paper	Time of development
Phenol-H_2O (5:1)	Aspartic acid, glutamic acid, serine, glycine, threonine, and alanine	Unbuffered	24 hrs.
o-Cresol saturated with pH 5 McILVAINE buffer	Methionine, valine, tyrosine, and phenylalanine	Buffered at pH 5	24
Benzyl alcohol-*n*-Butyl alcohol (1:1) saturated with pH 5 McILVAINE buffer	Phenylalanine, leucine, isoleucine, and proline	Buffered at pH 5	24
n-Butyl alcohol-Acetic acid-H_2O (4:1:5)	Cystine, and alanine	Unbuffered	24
sec-Butyl alcohol-3% Ammonia (3:1)	Histidine, arginine, and lysine	Unbuffered	24~30

acids from one another. The optimum temperature for their development was from 20 to 30°C.

After the development, the chromatograms run with alcohols were dried at room temperature and then at 60°C. The chromatograms run with phenols were dried at room temperature for 2 hours, subsequently washed with ether by dipping, and dried at 60°C. The dried chromatograms were dipped carefully in 0.5% ninhydrin solution in acetone and, after drying them in the air, heated at 75°C for 10 minutes to bring out all of the spots. When benzyl alcohol-butyl alcohol mixture was used as developer, the chromatogram was dipped two times in the ninhydrin solution and then heated.

Fig. 1. Paperchromatograms showing separation of amino acids by one-dimensional method.

Solvent mixtures: A. Phenol-H_2O (5:1); B. *o*-Cresol saturated with pH 5 McILVAINE buffer; C. Benzyl alcohol-*n*-Butyl alcohol (1:1) saturated with pH 5 McILVAINE buffer; D. *n*-Butyl alcohol-Acetic acid-H_2O (4:1:5); E. *sec*-Butyl alcohol-3% Ammonia (3:1).

In the determination of amino acids, the chromatograms of the sample and of standard amino acids are developed side by side in the same chromatographic glass trough under the same condition as described above.

3. Estimation of amino acids.

The coloured spots of individual amino acids developed on the standard and sample chromatograms were cut carefully from the filter paper. The colour was

eluted with 4 ml. of 75% alcohol and then 1 ml. of 0.1% $CuSO_4$ solution was added to it. The extinction of the developed salmon-pink colour was estimated with 500 mμ filter. When buffered filter paper was used in the development, 0.1% $CuSO_4$ solution dissolved in 0.1N H_2SO_4 was used, and when unbuffered filter paper the aqueous solution. The addition of $CuSO_4$ solution provided reproducible results. The intensity of colour was made stable for 30 minutes by addition of an aqueous solution of $CuSO_4$ but only for 15 minutes by addition of $CuSO_4$ dissolved in 0.1N H_2SO_4. Thus, the amino acid concentration is ready for calculation from the extinction of each spot both of standard and specimen.

4. Accuracy of the method.

When estimation was made with the mixture of amino acids of known concentrations, the recovery of each amino acid ranged from 95 to 103% except in the case of proline. The recovery of proline is not reproducible; therefore, this method cannot be employed for estimation of this amino acid.

5. Determination of amino acid composition of protein.

The samples corresponding to 20 to 30 mg of total N are hydrolyzed for 20 hours with 300 times of 6N HCl. The hydrolysate is concentrated to dryness *in vacuo* to remove HCl. The residues are dissolved in water (about 20 ml.) and neutralized to pH 5–6 with 0.5N NaOH, using pH test-paper of brom cresol green and methyl red. The hydrolysate is then fitered and the filter is washed thoroughly with water. The combined filtrate and washings are let subsequently to pass through a column of resin IRC-50 buffered at pH 4.7 with acetate buffer. The column is washed with water as completely as possible. The combined effluents are concentrated *in vacuo* to dryness. The residues are dissolved in water and made up to 10 ml. (monoamino acid fraction). The diamino acids, which are adsorbed on the resin, are eluted thoroughly with 1N HCl. The efflux is concentrated *in vacuo* to dryness. The residues are dissolved in water and made up to 10 ml. (diamino acid fraction). From 0.002 to 0.004 ml. portions of the sample solution and of the standard solution of each amino acid are applied to the paper with capillary pipette respectively and developed under the same condition as described above. The standard solution of each amino acid is prepared so as to contain 0.2 to 0.3γ of α-amino nitrogen in 0.001 ml. and preserved in refrigerator with chloroform when not in use. The standard solutions appear to be stable for at least a month. After development, the amino acid is estimated colorimetrically of its concentration as described before.

In the case of protein-hydrolysate the diamino acids resolved by solvent mixture *sec*-butyl alcohol-3% ammonia is somewhat incomplete and hence the estimated values for diamino acids may not completely be free from some errors. Thus, it may be advisable in the estimation of arginine and histidine rather to adopt other colorimetric method such as Sakaguchi- and Pauli's-methods using diamino acid fraction.

The present procedure seems to be an adequate for analysis of amino acid

composition of protein, although it is somewhat complicated in respect of its requiring several kinds of solvent mixtures.

Summary

An improved method for estimating amino acids by paperchromatography has been described. The method consists of the resolution of amino acids by a one-dimensional method and a subsequent colorimetric determination of ninhydrin colour. By the present method 16 amino acids are able to be determined with high accuracy.

References

1) McFarren, E. F.: *Anal. Chem.*, **23**, 168 (1951).
2) McFarren, E. F. and Mills, J. M.: *Anal. Chem.*, **24**, 650 (1952).
3) Maruta, Y., Samejima, H. and Tanaka, M.: *J. Agr. Chem. Soc. Japan*, **28**, 775 (1954).
4) Mizushima, S. and Akabori, S.: Chemistry of Proteins (in Japanese), Vol. 1, 38 (1954), Tokyo.
5) Roland, Jr., J. F., and Gross, A. M.: *Anal. Chem.*, **26**, 502 (1954).

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

EFFECT OF DIFFERENCES OF INITIAL AND FINAL TEMPERATURE OF WHALEMEAT WHEN IT IS TAKEN INTO AND OUT OF FREEZER ON DRIP

Kazuo Tanaka and Takeo Tanaka*

(Received Jan. 16, 1957)

Introduction

It is a fact that if a whalemeat is taken into a freezer with comparatively low initial temperature by precooling and out of the freezer with comparatively high final temperature, its freezing time will be shortened and a capacity of freezer will increase. But reduction of drip value and check of deterioration of the meat without decreasing the capacity of freezer are important problems given to us. Under such circumstances the authors researched into the effect of differences of initial and final temperature of the whalemeat when it is taken into and out of the freezer on drip during freezing. To obtain some proper measures to reduce the drip value and maintain the high quality authors carried out experiments.

Experimental

1. Three Types of Frozen Whalemeat.

It was already reported that three types of whalemeat, i.e. contractile, semi-contractile and non-contractile, can be observed as its frozen tissue is thawed. The contractile type frozen in pre-rigor stage gives a lot of drip showing obviously a feature of thaw-rigor with noticeable contraction of its tissues, the semi-contractile type frozen in full-rigor stage exudes not so much amount of drip showing to some extent the feature of thaw-rigor with slight contraction of its tissues, the non-contractile type frozen in post-rigor stage drips a small amount of the body fluids with no appreciable contraction, on thawing.

With the view that drip is exuded on thawing from frozen whalemeat due to the contraction of muscle tissues as well as the liberation of water from muscle protein due to the degeneration of protein during freezing, we have pursued the change in drip values in relation to the ATP level and the value of Protein N/Total N of the drip, taking the former as the index for the contractibility and the latter for the degeneration of protein hitherto. But in this experiment, the latter was not pursued.

2. Effect of Difference of Initial Temperature of Whalemeat When It Is Taken into Freezer on Drip.

Blocks of lean meat of fin whales caught in the Antarctic Ocean, both in pre-rigor stage and post-rigor stage were cut to a rectangular shape of about 12 cm × 30 cm × 39 cm in size and roughly 15 kg in weight. One of the test blocks

* Tokai Regional Fisheries Research Laboratory.

Table 1. Effect of difference of initial temperature of whalemeat when it is taken into freezer on drip.

C[1])—Contractile type: The samples of this type are frozen at the pre-rigor stage (pH value in fresh state 6.55 at +30°C of initial temperature and 6.05 at +5°C of initial temperature) and show contraction on thawing.

N[2])—Non-contractile type: The samples of this type are frozen at the post-rigor stage (pH value in fresh state 5.70 both at +30°C and +5°C of initial temperature) and show no contraction on thawing.

2.5 kg/cm²g[3])—Squeezing pressure: The expressible drip was exuded under pressure of 2.5 kg/cm²g after 24 hours exudation of free drip.

Initial Temp. °C	Volume Contraction %						Drip %						pH				Δ7P-P Content in free drip of contractile type mg %	Protein N / Total N ×100%			
	in 6 hrs.		in 24 hrs.		in 2.5 kg/cm²g[3])		in 6 hrs.		in 24 hrs.		in 2.5 kg/cm²g		in free drip		in exp. drip			in free drip		in exp. drip	
	C[1])	N[2])	C	N	C	N	C	N	C	N	C	N	C	N	C	N		C	N	C	N
+30 (Without precooling)	47.8	30.6	50.5	36.4	10.7	14.3	31.5	8.7	34.2	10.8	14.0	20.6	6.47	5.89	5.61	5.87	55.4	—	—	—	—
+5 (With precooling)	27.7	20.1	44.4	30.3	18.7	16.2	24.5	7.1	30.1	12.5	18.3	20.7	5.97	5.77	5.70	5.78	25.4	—	—		—

Table 2. Effect of difference of final temperature of whalemeat when it is taken out of freezer on drip.

C[1])—Contractile type: The samples of this type are frozen at the pre-rigor stage (pH value in fresh state 6.55) and show contraction on thawing.

N[2])—Non-contractile type: The samples of this type are frozen at the post-rigor stage (pH value in fresh state 5.70) and show no contraction on thawing.

2.5 kg/cm²g [3])—Squeezing pressure: The expressible drip was exuded under pressure of 2.5 kg/cm²g after 24 hours exudation of free drip.

Final Temp. °C	Volume Contraction %						Drip %						pH				Δ7P-P Content in free drip of contractile type mg %	Protein N / Total N ×100%			
	in 6 hrs.		in 24 hrs.		in 2.5 kg/cm²g[3])		in 6 hrs.		in 24 hrs.		in 2.5 kg/cm²g		in free drip		in exp. drip			in free drip		in exp. drip	
	C[1])	N[2])	C	N	C	N	C	N	C	N	C	N	C	N	C	N		C	N	C	N
−5 (Comparatively high)	34.8	24.1	13.7	24.2	46.0	26.6	32.7	6.0	33.1	9.9	17.6	18.7	6.09	5.83	5.94	5.77	31.7	—	—	—	—
−20 (Comparatively low)	38.2	24.8	38.8	28.9	18.2	20.7	33.4	5.4	34.7	7.2	12.8	21.9	6.27	5.84	5.64	5.81	41.5	—	—	—	—

was precooled first in air at about 0°C for 4 hours, and further in sea water at about −0.5°C for two hours while the other was not precooled. The temperature of the central part of the former was finally attained to +5°C or so but that of the latter still retained +30°C or so. Then the meats not only in pre-rigor stage and post-rigor stage but also with and without precooling were frozen in a multiplate type freezer by $CaCl_2$ brine of temperature −26°C for 6.5 and 8.5 hours or so respectively. After freezing, the frozen meats were glazed, packed in cartons and stored at −20°C or so for about three months, then, the test pieces, a rectangular shape of about 5 cm×10 cm×15 cm in size of roughly 0.75 kg in weight, were cut from them, and thawed in still air at +20°C. During thawing, both drip value and pH value of drip were measured on each test pieces. With test pieces of pre-rigor type, ATP level (shown by Δ7P-P mg/100 cc drip) was also measured. The results are given in Table 1.

In spite of remarkable difference observed between the contractile (pre-rigor) and non-contractile (post-rigor) types, marked difference were not found between the meats with and without precooling. The drip value of precooled meat of contractile type is to some extent less than that of non-precooled. Taking into consideration of the change of pH value and ATP level, it is presumed that the former was gradually turned to semi-contractile (full-rigor) type during precooling while the latter was not turned to. These experimental results lead us to the conclusion that to reduce the drip value, the whalemeat of contractile type should be frozen after it was converted into full-rigor or post-rigor type by precooling. And then, the low initial temperature of the meat before freezing will be contributable to diminish the freezing time of the meat.

3. Effect of Difference of Final Temperature of Whalement When It Is Taken out of Freezer on Drip.

Blocks of meat as above, both in pre-rigor stage and post-rigor stage were frozen in a multiplate type freezer by $CaCl_2$ brine of temperature −26°C until −5°C was the final temperature of the central part of the one and −20°C was that of the other, for 6.5 and 8 hours or so respectively. After freezing, the frozen meats were glazed, packed in cartons and stored at −20°C or so for about three months, then, the test pieces as above were cut from them, and thawed in still air at +20°C. During thawing, both drip value and pH value of drip were measured on each test pieces. With test pieces of pre-rigor type, ATP level (shown by Δ7P-P mg/100 cc drip) was also measured. The results are given in Table 2.

Again, in spite of remarkable difference observed between the contractile (pre-rigor) and non-contractile (post-rigor) types, marked difference could not be found between the meats with comparatively high and low final temperature. The drip value of meat of contractile type with comparatively high final temperature is to some extent less than that with comparatively low final temperature. Taking into consideration of the change of pH value and ATP level, it is presumed that the former was gradually turned to semi-contractile (full-rigor) type during subsequent cold storage while the latter was not turned to. In the frozen whalemeat with comparatively high final temperature, however, it is considered that the thickness of glazing was less than that with comparatively low final

temperature and the former was easily pressed out and deformed than the latter as it was stacked in cold storage. But we could not observe any deterioration on the quality of the former except the above defects. Therefore, to reduce the drip value, the whalemeat of contractile type would rather be taken out of the freezer as the final temperature is comparatively high, but to obtain the good quality, the whalemeat should be taken out of the freezer as the final temperature is as low as possible. These experimental results lead us to the conclusion that to reduce the drip value and obtain the high quality, the whalemeat should be taken out of the freezer after the final temperature of its central part has been reached at least −5°C or below.

Conclusion

The differences of initial and final temperatures of whalemeat when it is taken into and out of the freezer are not so affected on drip. However, it is recommended that the initial temperature of the meat should be as low as possible, to increase the capacity of the freezer and the final temperature of the meat should be at least −5°C or below, to decrease the deformation of the frozen meat after it was stacked in cold storage.

Acknowledgement

The authors express their heartfelt gratitude to Dr. K. Amano, Technical Official of the Tokai Regional Fisheries Research Laboratory, for his kind advices.

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

DRIP FROM FROZEN WHALEMEAT AFFECTED BY FREEZING RATE AND AIR TEMPERATURE IN AIR DEFROSTING

Kazuo Tanaka and Takeo Tanaka*

(Received Jan. 16, 1957)

Introduction

It is a well-known fact that frozen whalemeat yields a large amount of drip on thawing. The amount of drip depends largely on the freshness of the meat, partially on the storage condition and the manner of treatment before or after freezing of the meat, but it will be also affected by the freezing rate and the air temperature if being defrosted in air. The authors carried out experiments on these points.

Experimental

1. Feature of Frozen Whalemeat.

It was already reported that three types of whalemeat, i.e. contractile, semi-contractile and non-contractile, can be observed as its frozen tissue is thawed. The contractile type frozen in pre-rigor stage gives a lot of drip showing obviously a feature of thaw-rigor with noticeable contraction of its tissues, the semi-contractile type frozen in full-rigor stage exudes not so much amounts of drip showing to some extent the feature of thaw-rigor with slight contraction of its tissues, the non-contractile type frozen in post-rigor stage drips a small amount of the body fluids with no appreciable contraction, on thawing.

With the view that drip is exuded on thawing from frozen meat due to the contraction of muscle tissues as well as the liberation of water from the muscle protein due to the degeneration of protein during freezing and defrosting, we pursued the change in drip value in relation to the ATP level and the value of Protein N/Total N of the drip, taking the former as the index for the contractibility and the latter for the degeneration of protein.

2. Effect of Freezing Rate of Whalemeat on Drip.

Blocks of lean meat of fin whales caught in the Antarctic Ocean, both in pre-rigor stage and post-rigor stage were cut to a rectangular shape of about 12 cm × 30 cm × 39 cm in size and roughly 15 kg in weight, and frozen in an air freezer by still air of temperature −18°C for 24 hours as well as a multiplate type freezer by $CaCl_2$ brine of temperature −26°C for 8 hours. From these freezing time, the former is so-called slow-freezing and the latter quick-freezing. After freezing, the frozen meats were glazed, packed in cartons and stored at −20°C or so for about three months, then, the test pieces, a rectangular shape of about 5 cm × 10 cm × 15 cm in size of roughly 0.75 kg in weight, were cut from

* Tokai Regional Fisheries Research Laboratory.

Table 1. Effect of freezing rate of whalemeat on drip.

C[1]—Contractile type: The samples of this type are frozen at the pre-rigor stage (pH value in fresh state 6.55) and show contraction on thawing.

N[2]—Non-contractile type: The samples of this type are frozen at the post-rigor stage (pH value in fresh state 5.70) and show no contraction on thawing.

2.5 kg/cm²g [3]—Squeezing pressure: The expressible drip was exuded under pressure of 2.5 kg/cm²g after 24 hours exudation of free drip.

Freezing Time hrs.	Volume Contraction %						Drip %						pH				Δ7P-P Content in free drip of contractile type mg %	Protein N / Total N ×100%			
	in 6 hrs.		in 24 hrs.		in 2.5 kg/cm²g[3]		in 6 hrs.		in 24 hrs.		in 2.5 kg/cm²g		in free drip		in exp. drip			in free drip		in exp. drip	
	C[1]	N[2]	C	N	C	N	C	N	C	N	C	N	C	N	C	N		C	N	C	N
24 (Slow freezing)	25.2	10.7	29.7	2.7	23.4	56.7	20.7	4.3	25.9	7.8	22.0	24.5	6.05	5.71	5.84	5.85	17.4	—	—	—	—
8 (Quick freezing)	43.8	11.9	50.2	12.0	7.5	45.7	31.4	7.8	33.5	10.2	16.0	21.2	6.24	5.82	5.80	5.89	49.1	—	—	—	—

them, and thawed in still air at +20°C. During thawing, both drip value and pH value of drip were measured on each test pieces. With test pieces of pre-rigor type, ATP level (shown by Δ7P-P mg/100 cc drip) was also measured. The results are given in Table 1.

In spite of remarkable difference observed between the contractile (pre-rigor) and non-contractile (post-rigor) types, marked difference were not found between the slow-frozen and quick-frozen meats. The drip value of slow-frozen meat of contractile type is less in quantity than that of quick-frozen. Taking into consideration of the change of pH value and ATP level, it is presumed that the former was gradually turned to semi-contractile (full-rigor) type during slow-freezing while the latter was not turned to. These experimental results lead us to the conclusion that to reduce the drip value, regardless of the quality of the meat and the efficiency of the freezer, the whalemeat of contractile type would rather be slow-frozen, but non-contractile one should be quick-frozen to avoid the risk of producing meat with disgustful odour.

And then, after defrosting, the value of pH of the meat of non-contractile type was always higher than that of unfrozen state, on the contrary, in the meat of contractile type, as expected, it was always lower than that of unfrozen state. Such result, especially in non-contractile type, cannot be clearly explained at this experimental procedure.

3. Effect of Air Temperature in Air Defrosting on Drip from Frozen Whalemeat.

Blocks of meat as above, both in pre-rigor stage and post-rigor stage were frozen in a multiplate type freezer by $CaCl_2$ brine of temperature −26°C for 8 hours. After freezing, the frozen meats were glazed, packed in cartons and stored at −20°C or so for about three months, then, the test pieces as above were cut from them, and thawed in still air at +5, +10, +20 and +30°C of air temperature respectively for hours. During thawing, the values of drip, pH and Protein N/Total N in drip were measured on each test pieces. With test pieces of pre-rigor type, ATP level (shown by Δ7P-P mg/100 cc drip) was also measured. The results are given in Table 2.

Remarkable differences between the meats kept in +5, +10°C of air temperature and that of +20, +30°C of air temperature were observed on both contractile (pre-rigor) and non-contractile (post-rigor) types. The drip values from the frozen meats defrosted in +5, +10°C of air temperature are less than those defrosted in +20, +30°C of air temperature. But the defrosting time of the former was about 20∼10 hours while that of the latter about 6∼4 hours. From these defrosting time, the former is so-called slow-defrosting and the latter quick-defrosting. It can be pointed out, therefore, that the slow-defrosting gives to some extent less quantity of drip and good quality of defrosted meat than the quick-defrosting. The drip value from the frozen meat of non-contractile type defrosted in +10°C of air temperature is somewhat less than that in +5°C of air temperature. These experimental results lead us to the conclusion that to reduce the drip value and obtain the high quality of the defrosted meat, the best defrosting process of frozen whalemeats both contractile and non-contractile types is still

Table 2. Effect of air temperature in air defrosting on drip from frozen whalemeat.

C[1]—Contractile type: The samples of this type are frozen at the pre-rigor stage (pH value in fresh state 6.65) and show contraction on thawing.

N[2]—Non-contractile type: The samples of this type are frozen at the post-rigor stage (pH value in fresh state 5.70) and show no contraction on thawing.

2.5 kg/cm²g[3]—Sqeezing pressure: The expressible drip was exuded under pressure of 2.5 kg cm²g after 24 hours exudation of free drip.

Air Temp. °C	Defrosting Time hrs.	Volume Contraction %		Drip %		pH		ΔTP-P Content of contractile type	Protein N / Total N ×100 %	
		C[1]	N[2]	C	N	C	N	mg %	C	N
	0	0.0	0.0	0.0	0.0	—	—	—	—	—
	1	0.0	0.0	0.0	0.0	—	—	—	—	—
	2	0.0	0.0	0.0	0.0	—	—	—	—	—
	3	0.0	0.5	0.0	0.0	—	—	—	—	—
	4	0.0	1.2	0.0	0.0	—	—	—	—	—
	5	0.0	1.8	0.0	0.0	—	—	—	—	—
+5	6	0.0	2.5	2.7	1.1	5.95	5.76	19.3	70.6	—
	7	0.0	4.9	3.9	1.7	5.90	5.86	—	77.1	—
	8	0.0	7.1	7.1	2.8	5.90	5.82	21.2	64.3	78.9
	9	7.4	8.9	8.0	3.8	5.85	5.71	—	72·5	76.1
	10	18.2	15.6	9.4	4.7	5.84	5.69	13.7	75.0	63.7
	24	34.2	12.1	21.1	13.0	5.76	5.62	—	77.8	72.3
	2.5 kg/cm²g[3]	15.5	42.6	27.4	21.8	5.59	5.68	—	59.3	63.0
	0	0.0	0.0	0.0	0.0	—	—	—	—	—
	1	0.0	0.0	0.0	0.0	—	—	—	—	—
	2	0.0	2.4	0.0	0.0	—	—	—	—	—
	3	1.2	2.4	2.9	1.6	6.02	5.71	25.9	—	68.3
	4	2.7	3.3	5.2	3.1	5.95	5.78	—	69.4	71.1
	5	4.2	5.9	8.9	4.9	5.90	5.70	16.7	70.4	73.1
+10	6	5.4	8.1	12.3	6.9	5.91	5.71	—	69.1	72.6
	7	6.1	8.1	15.9	8.3	5.91	5.72	11.6	72.2	—
	8	7.2	7.5	18.4	9.5	5.89	5.68	—	71.1	76.1
	9	11.4	8.8	21.2	10.5	5.81	5.64	—	72.2	91.5
	10	15.5	5.9	23.4	11.2	5.80	5.60	—	68.5	—
	24	31.4	12.2	32.2	15.7	5.51	5.60	—	66.3	78.6
	2.5 kg/cm²g	9.6	31.2	15.7	14.1	5.61	5.71	—	44.7	61.9
	0	0.0	0.0	0.0	0.0	—	—	—	—	—
	1	5.4	1.2	1.5	0.8	6.26	—	34.0	65.4	—
	2	10.2	2.4	8.0	3.0	6.14	5.80	—	67.4	74.6
	3	15.3	6.1	15.7	6.1	6.01	5.73	36.0	72.8	88.0
+20	4	20.5	9.3	21.6	8.7	5.82	5.75	—	70.1	81.2
	5	22.1	14.2	29.5	10.9	5.81	5.70	20.0	73.1	79.8
	6	24.4	20.0	33.3	12.0	—	5.70	—	—	—
	24	39.1	27.4	35.6	25.4	6.04	5.88	—	63.3	66.0
	2.5 kg/cm²g	16.3	26.4	12.5	6.8	5.91	6.13	—	64.7	64.8
	0	0.0	0.0	0.0	0.0	—	—	—	—	—
	1	10.2	4.8	4.7	3.2	6.20	5.72	55.0	64.1	76.6
	2	18.3	9.4	12.3	7.4	6.05	5.73	—	71.2	82.4
	3	21.5	14.2	19.2	11.0	5.89	5.72	40.0	70.3	77.4
+30	4	26.5	17.3	27.1	14.2	5.83	5.66	13.0	73.1	74.7
	5	30.4	21.9	32.3	16.0	—	—	—	—	—
	6	33.3	26.8	35.1	17.6	—	—	—	—	—
	24	46.2	23.3	35.8	25.2	5.86	5.66	—	68.7	68.9
	2.5 kg/cm²g	10.4	30.9	11.4	8.9	5.89	5.96	—	62.6	62.8

air defrosting in +10°C or so of air temperature. The reason of this principle may be interpreted as below:

In contractile type, the velocity of the decline in ATP level is greater than that of the defrosting of the body juice in frozen tissue in +5°C, +10°C of air temperature, while the former is smaller than the latter in +20°C, +30°C of air temperature. By defrosting the frozen whalemeat of this type at +5°C, +10°C of air temperature, therefore, its contractibility fades away more rapidly than the thawing of the frozen tissue. And the defrosting in +10°C of air temperature is superior to that in +5°C of air temperature because of its quicker defrosting speed.

In non-contractile type, on one hand, it may be considered that the slow-defrosting gives better quality of defrosted meat and less quantity of drip from frozen tsssue than the quick-defrosting, because the slower the frozen tissue is defrosted, the more perfect of reproducibility of the separated muscle fibre of the tissue is. On the other hand, it may be also considered that the quick-defrosting gives better quality of defrosted meat and less quantity of drip from frozen tissue than the slow-defrosting, because the quicker the frozen tissue is defrosted, the less denature the muscle protein is (the frozen meat passes through quickly the zone of maximum crystal formation: −5∼−1°C in quick-defrosting). These two theories are supposed true though they are contradictory to each other at a glance. By the defrosting speed of the frozen whalemeat of this type at +10°C or so of a moderate air temperature, therefore, it may give an intermediate one of these two defrosting speed.

With the drip values from the frozen whalemeat given by both this and previous reports, it is presumed that the amount of drip depends largely on the freshness of the meat and somewhat the air temperature in air defrosting, while it is seldom affected by the freezing rate, the storage condition and the manrer of treatment before or after freezing of the meat.

Conclusion

To obtain the frozen whalemeat with small amount of drip and superior quality, it is recommended that the whalemeat of contractile type should be slow-frozen to remove the contractibility during freezing while that of non-contractile type should be quick-frozen.

The amount of drip from the frozen whalemeat depends largely on the freshness of the meat, and next to on the air temperature in air defrosting while it is seldom affected by the freezing rate. Recommended air temperature in still air defrosting is practically +10°C or so.

Acknowledgement

The authors express their heartfelt gratitude to Dr. K. Amano, Technical Official of the Tokai Regional Fisheries Research Laboratory, for his kind advices.

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

POLAROGRAPHIC STUDIES ON CUTTLE FISH MEAT. PART I.

Isami Osakabe, Kiyoshi Fukushima, Ikunosuke Okada and Masao Hasegawa

(Received Jan. 10, 1957)

On the polarographic studies[1] of the fish meat and its viscera, an oxidation wave (see Fig. 1) of SH-group was obtained, but no reduction wave was obtained as shown in Fig. 2, at the range of −1.0 to −1.7 volt. However, the authors have obtained a characteristic reduction wave in the water soluble extract of cuttle fish meat as shown in Fig. 3.

Then the polarographic reduction wave as shown in Fig. 3 was assumed to be a characteristic constituent in the cuttle fish meat. The authors have tried to investigate the properties of this constituent of cuttle fish meat which shows a reduction wave.

Fig. 1. Polarogram of the water soluble extract of carp fish meat, the supporting electrolyte was McIlvaine buffer (pH = 7.0). SH-oxidation potential, −0.35 volt. vs. S.C.E.

Experiment

I. Solubility.

The constituent showed a reduction wave was readily soluble in water, slightly souble in alcohol, and insoluble in ether.

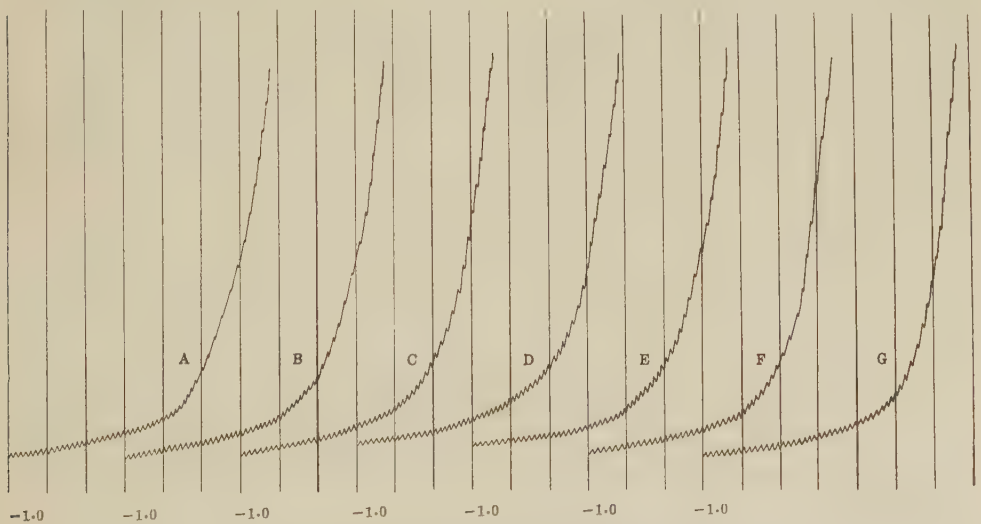


Fig. 2. Polarograms of various fish meats.
A, *Trachurus japonicus*; B, *Sardinops melanosticta*; C, *Scomber japonicus* (dark-coloured flesh); D, *Scomber japonicus*; E, *Seriola quinqueradiata*; F, *Cololabis saila*; G, *Pleurogrammus azonus*.

II. Results by steam distillation.

When the water soluble extract of cuttle fish meat was steam-distilled, the substance showed a reduction wave was obtained in the residual fraction, but not in the distillate as shown in Fig. 4.

From above results, it was recognized that this substance might have been nonvolatile and heat-stable.

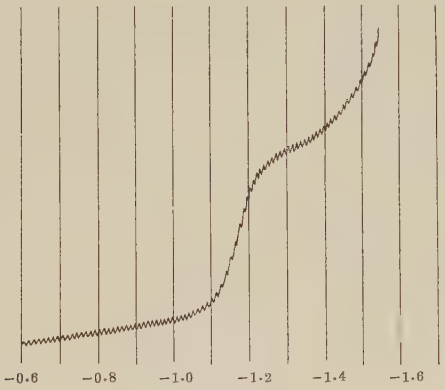


Fig. 3. Polarogram of the water soluble extract of cuttle fish meat, the supporting electrolyte was McIlvaine buffer (pH=7.0).

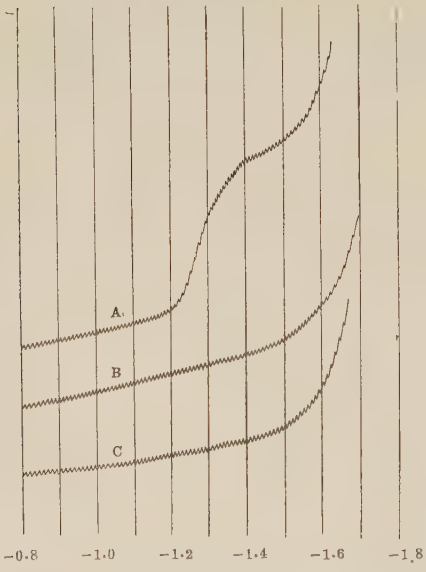


Fig. 4. Polarograms of the steam distillate and the residual fraction of the water soluble extract of cuttle fish meat in McIlvaine buffer solution (pH=7.0). A, Residual fraction; B, Steam distillate fraction; C, McIlvaine buffer solution (pH=7.0) as a supporting electrolyte.

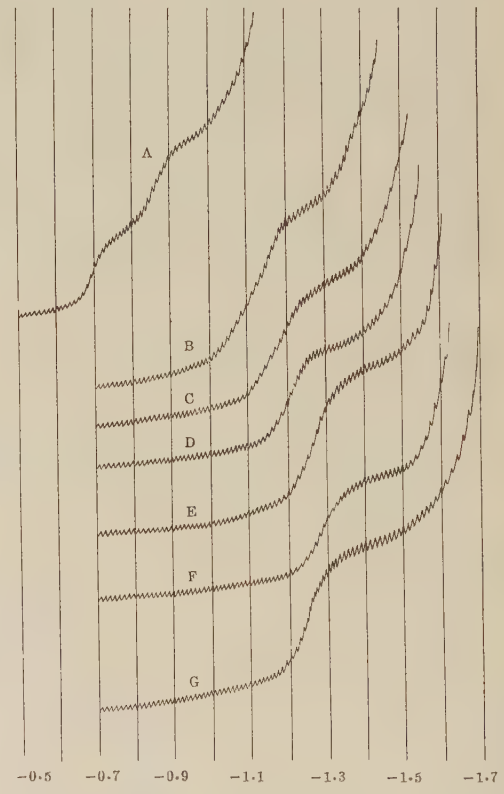


Fig. 5. Effect of pH on half-wave potential (vs. S.C.E.) A, −0.81 V (1st wave), −0.96 V (2nd wave); B, −1.12 V (pH=4.0); C, −1.18 V (pH=4.8); D, −1.20 V (pH=6.0); E, −1.27 V (pH=7.0); F, −1.29 V (pH=8.0); G, −1.24 V. Supporting electrolytes of each polarograms were as follows, A, 1 N-H_2SO_4 soln.; B—F, McIlvaine buffer series; G, 0.1 N-KCl soln.

III. Effect of pH of supporting electrolytes on the reduction potential.

The potential of the polarographic reduction wave was influenced by pH of supporting electrolytes as shown in Fig. 5. The reduction potential was effected by pH of supporting electrolyte, the lower the pH, the more positive it was removed.

IV. Influences of freshness of cuttle fish meat.

The polarographic behavior and volatile basic content of cuttle fish meat were shown in Table 1, and Fig. 6, when the sample was allowed to stand at room temperature (3~11°C).

Table 1. Influences of the freshness of cuttle fish meat.

Days	NH_3-N	pH	Height of the wave	Reduction potential
	mg/100g		mm	volt.
0	19.54	6.1	27.0	−1.28
1	25.23	6.0	29.0	−1.28
2	35.82	6.5	37.5	−1.27
3	73.26	7.6	36.0	−1.30
5	248.10	8.0	25.0	−1.29

It seemed to be that the heights of each waves were almost definite, and independent on the volatile basic content.

V. Separation of the constituents of water soluble extract of cuttle fish meat by ion-exchange resins.

In order to separate organic acids from the other constituents in cuttle fish meat, the extracted-solution of sample was treated with ion-exchange resins as shown in Fig. 7.

V(a): Explanation of ion-exchange technique in Fig. 7.

i) Fraction 3. A 50 ml. of filtrate dealt with active carbon was added and allowed to run into the column, IRA-400 resin, with the flow rate of 1.0, 1.5 ml. per min., and after the operation of effluence was finished, the column was washed with 10 ml. of distilled water. The effluent from the column was made to be together with the washings from it.

ii) Fraction 4. Adsorbed substances with IRA-400 were eluted with 25 ml. of N–Na_2CO_3 solution. Organic acids might have been contained in its eluate as their sodium salts. Na-free organic acids were obtained in the filtrate, fraction 4, by the use of IR-120 resin. The methods for the effluence or the washing were the same as above described.

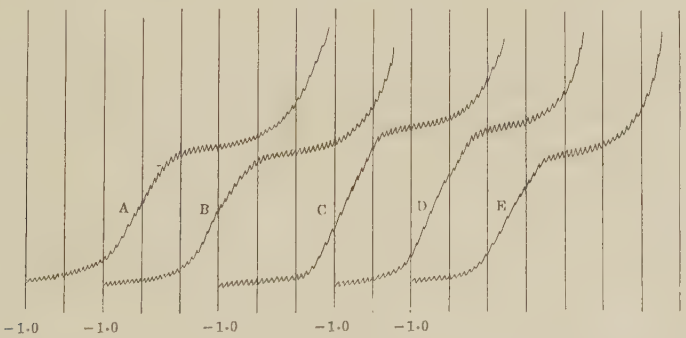
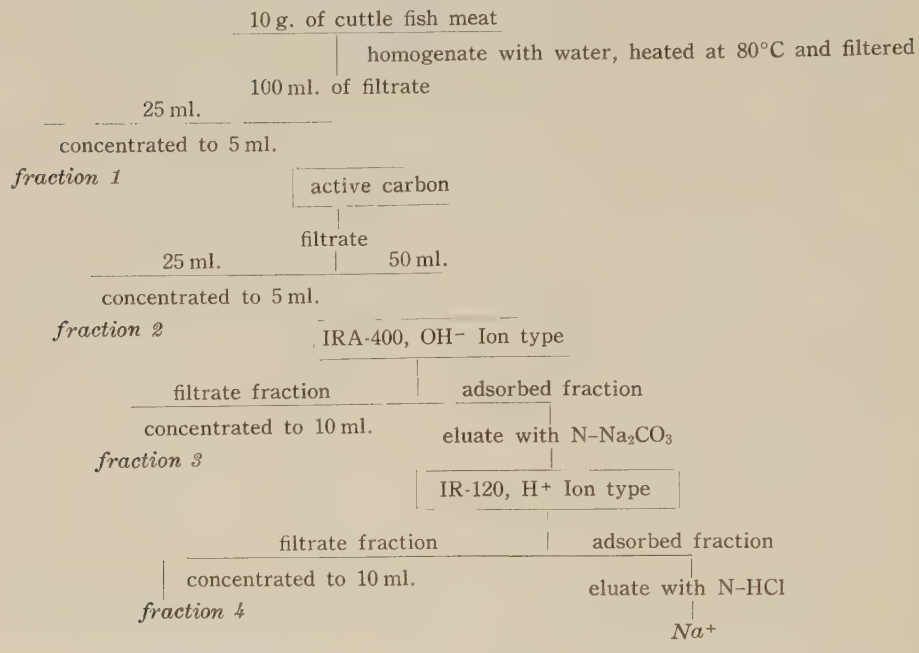


Fig. 6. Polarograms of cuttle fish meat-extract at the various freshness. A 2g of sample was homogenized with 10 ml. of water and filtered, and then 6 ml. of McIlvaine buffer (pH=7.0) was added to 2 ml. of the filtrate. Days stored were as follows. A, 0; B, 1; C, 2; D, 3; E, 5 days.

Fig. 7. Separation method (I) by ion-exchange resins[2].



V(b): Preparation of sample for the polarographic measurements.

Each filrates were concentrated to a fifth volume of original solution before filtration of effluence, and 18 ml. of buffer solution was added to 2 ml. of concentrated filtrates. Polarograms of fraction 1, 2, 3, and 4 were shown in Fig. 8.

As the above results, it was evident that the reduction waves were obtained in fractions 1 and 2 as shown in Fig. 7.

The adsorption with active carbon was fairly observed from the fact that height of the reduction wave of fraction 2 was lower than that of fraction 1.

It was easily expected that the reduction wave was not due to organic acids in cuttle fish meat, since they might have been almost included in fraction 4. On the other hand, the reduction wave was obtained, in fraction 3 in which almost various amino acids, organic bases, and sugars might have been probably included, and so the further separation methods were needed and developed by ion-exchange technique as follows.

VI. The further separation of the fraction 3 in Fig. 7.

The solution of fraction 3 was again passed through the column of ion-exchange resin, IR-120, and separated into the filtrate and adsorbed fraction. Then each fractions were examined by polarographic method. Consequently, no reduction wave was obtained in the filtrate in which some sugars might have been contained, and the substance showed a reduction wave was adsorbed strongly with the resin, IR-120.

When the adsorbed substances were eluted with 2N-ammonium hydroxide soln., the reduction wave was obtained in the eluate as shown in the polarogram

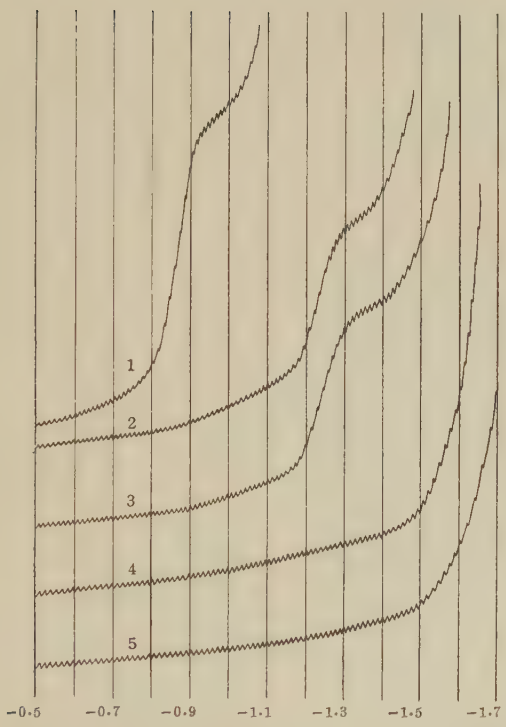


Fig. 8. Polarograms of each fractions shown in Fig. 7. 1, filtrate without active carbon, fraction 1; 2, filtrate with active carbon, fraction 2; 3, filtrate from IRA-400 resin, fraction 3; 4, adsorbed substances with IRA-400 resin, fraction 4; 5, McIlvaine buffer solution was used as the supporting electrolyte.

Fig. 9. Further investigation on the fraction 3 in Fig. 7 by polarographic method. A, Polarogram of filtrate fraction 3 in Fig. 7; B, Polarogram of adsorbed fraction with IR-120 resin, or of its fractional eluate; C, Polarogram of filtrate fraction from IR-120 resin in which sugars might have been contained.

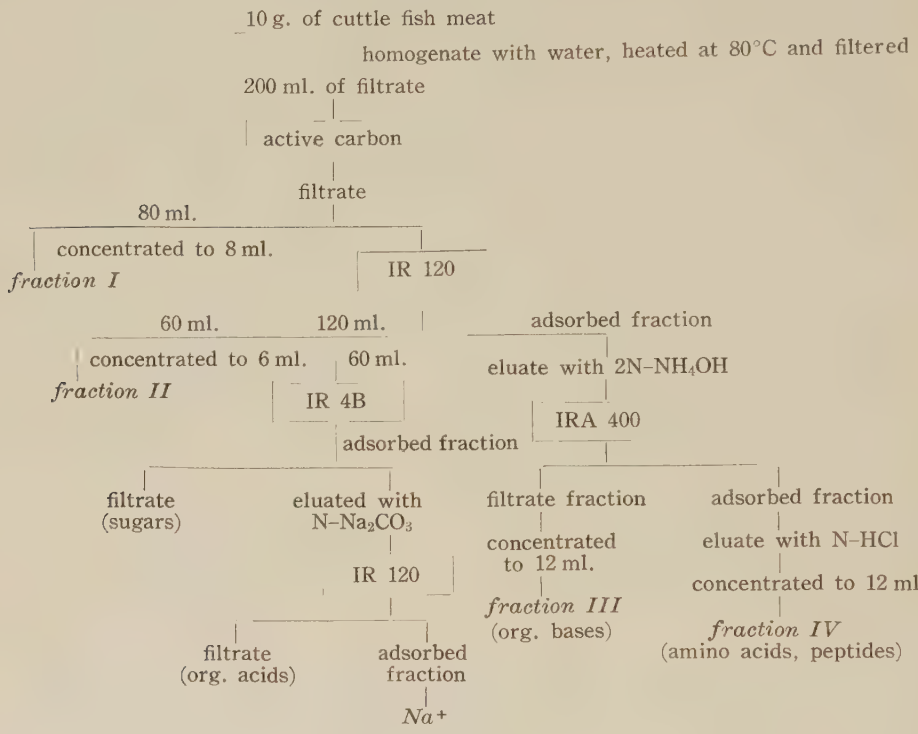
B, Fig. 9. Amino acids and bases were assumed to be contained in the eluate.

VII. Systematic further fractional separation with ion-exchange resins.

For the separation of organic bases from amino acids, IR-120 and IRA-400 resins were mainly used. Investigations on the other compounds in cuttle fish meat extract were carried out simultaneously. Details were shown in Fig. 10, and the methods for fractional separation were much the same as above mentioned in Fig. 7. Polarograms of fractions I, II, III, and IV were shown in Fig. 11.

The reduction wave was not obtained in fraction II (sugars and organic acids), and also in fraction IV (amino acid and peptides). While, only in fraction III (organic bases) it was obtained. Consequently, it was expected that the constituent showed a reduction wave in cuttle fish meat exract was probably a certain organic base substance. Then, further investigations were developed as follows.

Fig. 10. Further systematic separation method (II) of the constituents in cuttle fish meat by ion-exchange resins.



VIII. Studies on the organic base fraction by polarographic, spectrophotometric and paper-chromatographic methods.

So far as the above investigations were concerned, the polarographic reduction wave seemed to be due to a certain organic base. Therefore, several organic bases were probably contained in fraction III (Fig. 10), the following paper-chromatographic method was applied to isolate the constituent in it from the other substances. As a preliminary experiment, each fractions in Fig. 10 were investigated chromatographically in order to make sure of ion exchange capacity. In this experiment, wide strips, Whatman No. 1 papers, were used, and after simultaneous developments of chromatograms for each fractions, strips were dried and sprayed with ninhydrin as an indicator. There were 8 spots in fraction I, and IV, and 1 or 2 spots in fraction II and III. Most of free amino acids in cuttle fish extract were included in fraction IV, and excluded from fraction III or II. Thus the authors were able to make sure of ion-exchange capacity indirectly,

Then, studies on organic base fraction were carried out as follows. A wide sheet of paper (30 cm. in the width; 40 cm. in the height) was used, and a line was marked at a distance of 4 cm. from the edge of paper with pencil.

A 0.1 ml. of sample solution, organic base fraction III, was pipetted and

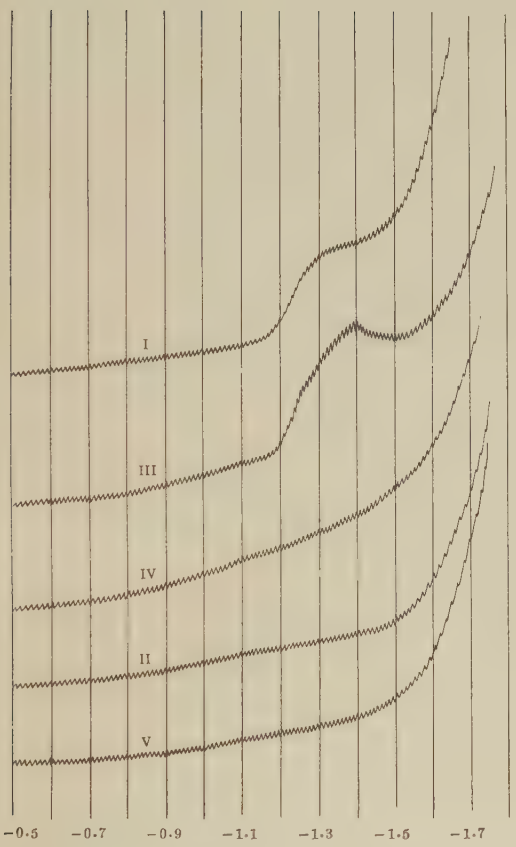


Fig. 11. Polarograms of each fractions in Fig. 10. I, fraction I; II, fraction II; III, fraction III; IV, fraction IV; V, McIlvaine buffer (pH=7.0).

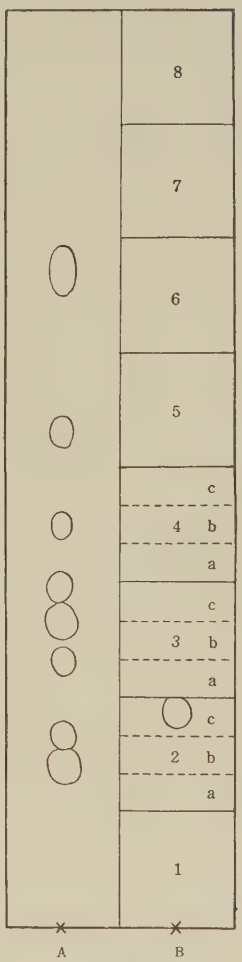


Fig. 12. Chromatograms of the amino acids, peptides, and organic bases fraction. A, Amino acids fraction; B, Organic base fraction. Rf of fraction 1= 0.000−0.122, Rf of fraction 2=0.122−0.245, Rf of fraction 3=0.245−0.367, Rf of fraction 4=0.367−0.490, Rf of fraction 5=0.490−0.612, Rf of fraction 6=0.612−0.735, Rf of fraction 7=0.735−0.857, Rf of fraction 8=0.857−1.00.

applied lineally on the line of paper. After drying the filter paper, above the same operation was repeated three times, and the sample solution pipetted was amount to 0.3 ml. Then the sample was developed with the solvent of *n*-butanol-acetic acid-water (4:4:1). After drying the paper in air, it was furthermore dried in an air oven to eliminate the solvent completely. The solvent was elimi-

nated throughly, then the paper was cut off from the starting line to the solvent front at the interval of 3 cm., and eight pieces, No. 1~No. 8, of filter paper were obtained. Then each pieces of paper were extracted with 6 ml. of McIlvaine buffer (pH=7.0) overnight. Extracted solutions from the each fractions (No. 1~8) were investigated by polarographic and spectrophotometric methods. And the observation was made on the relation between the reduction wave and ultra-violet

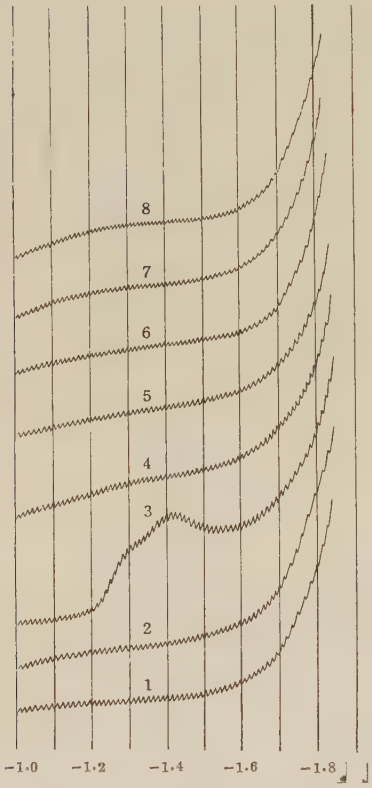


Fig. 13. Polarograms of each fractions. 1, McIlvaine buffer (pH =7.0); 2, No. 2. fraction; 3, No. 3. fraction; 4, No. 4. fraction; 5, No. 5. fraction; 6, No. 6. fraction; 7, No. 7. fraction; 8, No. 8. fraction. Each pieces of paper were extracted with 6 ml. of buffer, and the solution was examined by polarographic method.

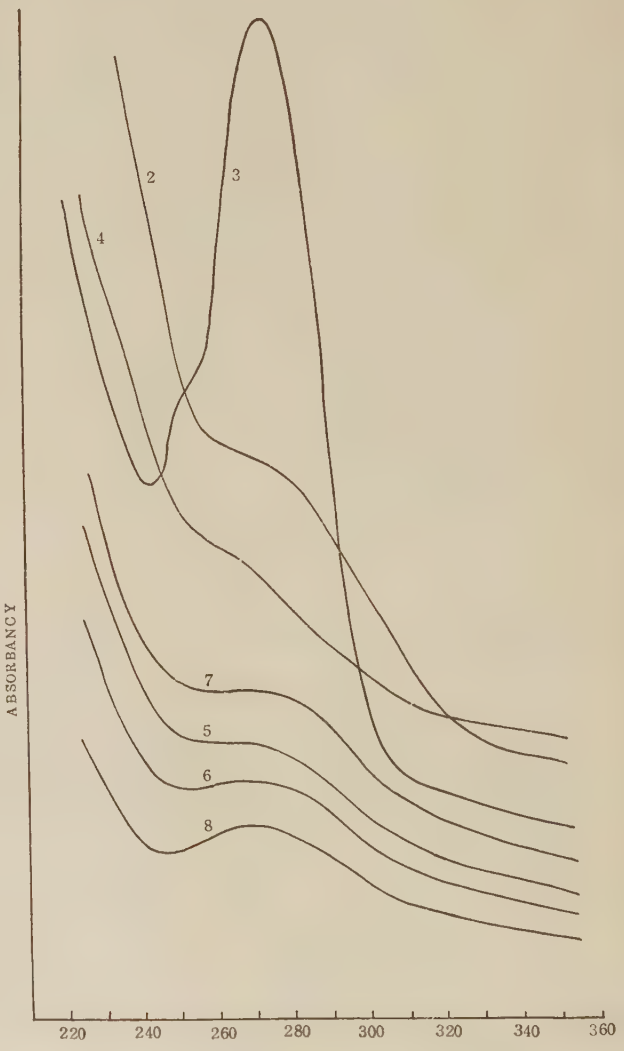


Fig. 14. Absorption spectrograms of each fractions. λmax=272 mμ. 2, No. 2. fraction; 3, No. 3. fraction; 4, No. 4. fraction; 5, No. 5. fraction; 6, No. 6 fraction; 7, No. 7. fraction; 8, No. 8. fraction. Distilled water was used as the extraction solvent, and the reading of absorbancy had been taken against distilled water, control.

absorption maximum. The reduction wave and absorption maximum were obtained in the 3rd zone, No. 3 (Fig. 12), as shown in Figs. 13 and 14.

Then the constituent showed a polarographic reduction wave was developed on the zone of Rf range, 0.24~0.36, with the solvent above mentioned. Since a colored spot was not found in the zone of No. 3, as shown in the chromatogram of organic base fraction, (see Fig. 12), NH_2-groups might have not been contained in the molecule of this substance showed a reduction wave.

Recovery test from the fraction No. 3 was shown in Fig. 15.

Height of the wave of extracted solution from the fraction No. 3 was almost the same as that of original sample. Then, the substance showed a reduction wave was almost recovered

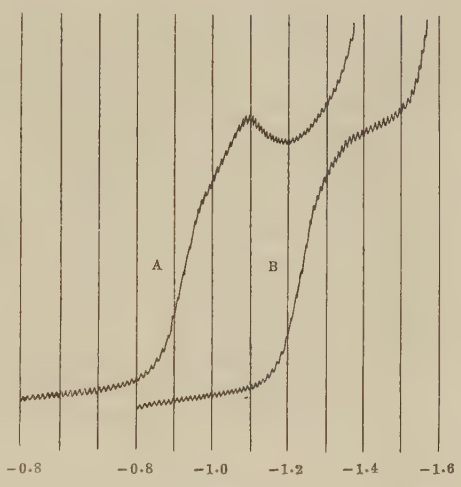


Fig. 15. Polarographic recovery test from the paper. A, Polarogram of the sample extracted from the fraction No. 3. (h=52.0 mm); B, Polarogram of original sample before development of chromatogram (h=53.5 mm).

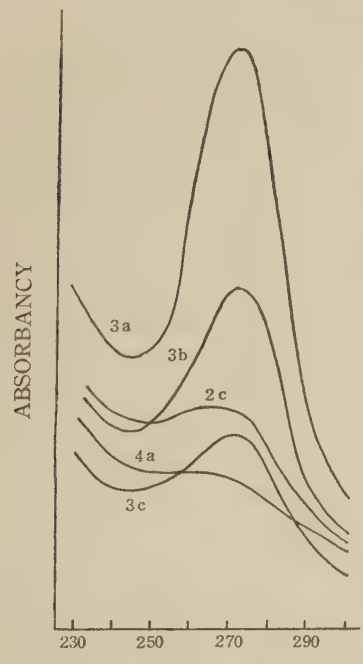


Fig. 16. Absorption spectrograms of the each subdivided fractions, 2c, 3a, 3b, 3c, and 4a show the positions on the strip of paper.

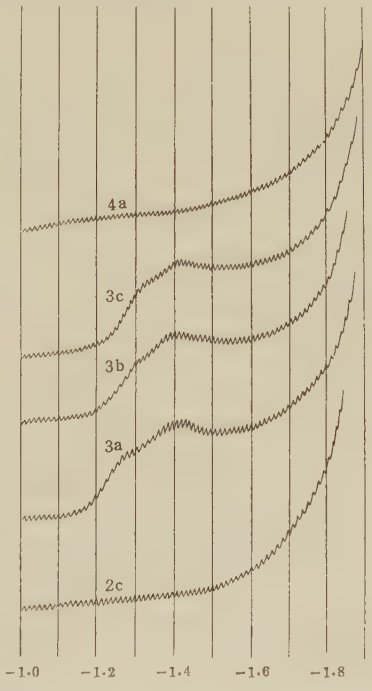


Fig. 17. Polarograms of the each subdivided fractions, 2c, 3a, 3b, 3c, and 4a show the positions on the strip of paper.

satisfactorily from the paper. The further investigations were made in details by chromatographic method on the zones below and above the No. 3 fraction, with the above method. And the filter paper was cut in nine small pieces, 2a, 2b, 2c, 3a, 3b, 3c, 4a, 4b, and 4c. The width of their each pieces was 1 cm. The results were shown in Figs. 16 and 17.

It was confirmed chromatographically that the constituent showed a reduction wave in cuttle fish meat extract had been developed in the range of Rf= 0.24~0.36. As shown on the fraction 3a, 3b and 3c in Figs. 16 and 17, the height of reduction waves were proportional to the optical density at 272 mμ. The constituent showed a reduction wave was isolated purely by the chromatographic method, and we were able to find that the constituent had been the same substance showed the absorption maximum, 272 mμ.

IX. Studies on the several authentic organic bases.

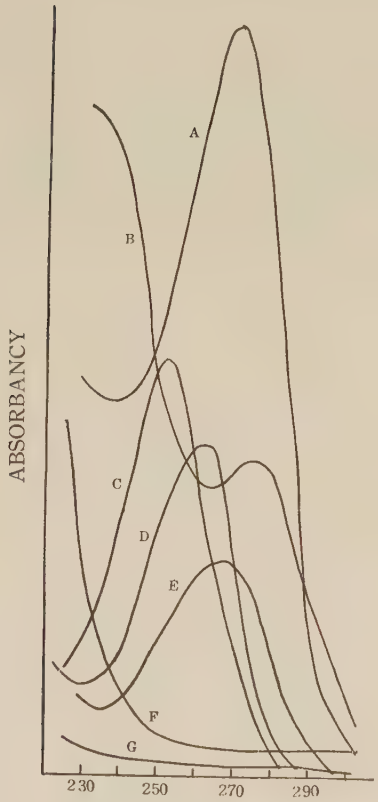


Fig. 18. Absorption spectrogram of several authentic organic bases. Distilled water was used as each solvents. Concentrations of each organic bases were about 10γ per ml. A, Xanthine; B, Thiamine; C, Hypoxanthine; D, Adenine; E, Thymine; F, Betaine; G, Choline.

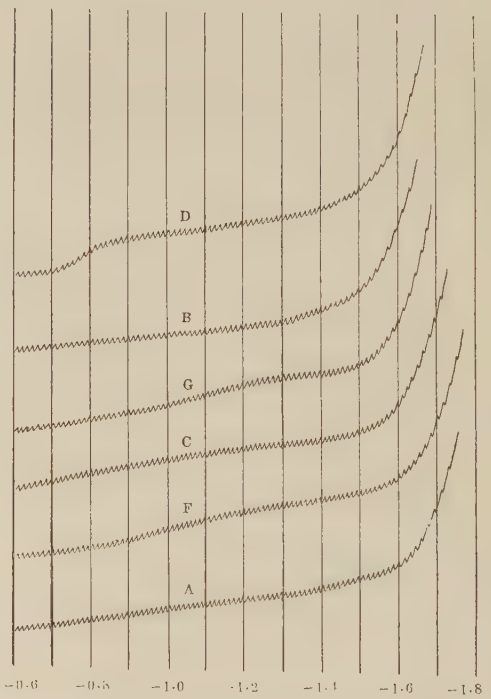


Fig. 19. Polarograms of several authentic organic bases. Buffer was used as supporting electrolyte. Concentrations of each organic bases were about 2×10^{-3} *M*. A, Xanthine; B, Thiamine; C, Hypoxanthine; D, Adenine; F, Betaine; G, Choline.

Since the constituent showed a reduction wave was confirmed to be an organic base which shows absorption maximum, 272 mμ, then the observations were carried out on the several authentic organic bases by polarographic and spectrophotometric methods. The results were shown in the Figs. 18 and 19.

The absorption maximum of xanthine was 270 mμ, and that of the constituent in cuttle fish meat was 272 mμ in the solvent of distilled water. The both absorption maximums were closely resembled, but the constituent was different from xanthine as the polarographic investigations, because the reduction wave was not obtained in xanthine, as shown in Fig. 19. It was confirmed that the constituent showed the reduction wave in cuttle fish meat extract was a certain organic base substance which showed the absorption maximum, 272 mμ.

Authors have also found that the constituent showed the reduction wave was included completely in the basic fraction by ion exchange resins. And also, the constituent showed the reduction wave was completely different from the above several authentic organic bases in their polarographic behavior. And further identification of the structure of this constituent is now under studying.

Résumé

(1) In the water soluble extract of the cuttle fish meat, a constituent showed a characteristic refined reduction wave was found by polarographic method.

(2) The properties of this constituent were as follows:

i) Readily soluble in water, alcohol and insoluble in ether.

ii) Stable to heat up to 100°C.

iii) Nonvolatile substance.

(3) It was recognized to be a certain organic base by ion exchange technique.

(4) As the results of the chromatogram of organic base fraction with the solvent, butanol-acetic acid-water (4:4:1), the constituent showed the reduction wave was developed in the range of Rf=0.24~0.36.

(5) The constituent showed a reduction wave was studied by spectrophotometric method, and its absorption maximum was 272 mμ.

(6) It was assumed that no amino groups were contained in the molecule of this constituent as the results of chromatographic investigations, and of ninhydrin reaction.

(7) The reduction wave was not influenced by the freshness of the sample.

(8) It was found that this constituent was not anyone of betaine, purine or pyrimidine bases, as the results of polarographic investigations.

Acknowledgement

Finally, authors express our sincere gratitude to Prof. Dr. I. Tachi of the Kyoto University, for his invaluable advice in this experiment. This study was supported in a part from the Ministry of Education, for which authors express hearty thanks.

References

1) Okada, Osakabe, Kuratomi and Kikuchi: J. of the Tokyo Univ. of Fish., **39**, 1, 31–38 (1952).

2) B. T. Overell: Nature., **167**, 361 (1951).

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

ENZYMATIC STUDIES ON THE GLYCOLYSIS OF FISH MUSCLE
I. ACTIVITY OF PHOSPHORYLASE

Toyoki Ono, Fumio Nagayama, Hirozo Osawa*
and Bunichro Takahashi**

(Received Jan. 16, 1957)

The *post mortem* spoilage of fish flesh seems to be distinguished into two types. The one is a chemical reaction, either enzymatic or non-enzymatic, and the other is caused by microorganisms.

Previous investigations concerning the freshness of fish flesh have dealt almost exclusively with the analytical results of the chemical components of fish muscle extract and scarcely been extended to the enzymatic examination.

That there have been little attempt to the enzymological investigation of the fish muscle may be attributed to the fact that the enzyme action of muscle of fish *post mortem* is exhibited and inactivated more rapidly than that of the mammals, as well as to the fact that it is very difficult to get so much specimen of live fish that the sufficient investigations are smoothly carried out.

Yamada *et al.*[1,2,3] made an interesting series of investigation from which they suggested a measure of freshness for some marine fishes resuming *post mortem* changes of some chemical components such as lactic acid, reducing sugar, organic and inorganic phoshpates, and phosphoglyceric acid. Amano *et al.*[4] and Fujimaki *et al.*[6] studied the chemical changes, especially the glycolysis in the flesh of fish killed in two different manners, and found that the glycolysis proceeded with special rapidity in the flesh of fish being struggled to death and not so rapidly as in the one being not struggled.

However, it seems desirable to ascertain the nature of the enzyme actions which occur in *post mortem*. The present investigation deals with the role of phosphorylase in the glycolysis of fish muscle being expected to provide some clues to the determination of the freshness of fish.

Experiments

I. Material and Methods

(1) Material

The young live fish of Japanese sea bass, Fukko (*Lateorabrax japonicus*) caught at the coast of Shinagawa, Tokyo Bay, of about 20 cm. in body length, was chosen as the sample for the present investigation. The fish was cultured in a tank for 3 hours in order to arrive at a certain physiological condition.

(2) Preparation of the enzyme solution

After the beheading followed by rapid out-flow of blood of a live fish,

* Present address, Nichiro Fisheries Co. Ltd., Kurihama, Yokosuka.
** Present address, Matsumoto Can Mfg. Co. Ltd., 12 Honjo-Takekawacho-1, Sumida-ku, Tokyo.

skin, bones, and dark muscle were removed. The flesh was ground two times by a chopper cooled with ice, and 5 g. of this blend was crushed further for 30 seconds with 25 ml. of buffer solution in a homogenizer driven in a cool. The crude enzyme solution was obtained as a clarified supernatant solution by the centrifugation of the homogenate at $1500 \times g$. for 10 minutes.

(*3*) *Factors in the preparation exerting a high enzyme activity*

(i) Effect of time for crushing

By a homogenizer each 5 g. of the flesh was crushed with 25 ml. of 0.2 M veronal buffer (pH 6.4), for 30, 60 and 180 seconds respectively and the hono-genates were centrifuged. The solutions obtained here were analyzed for glycogen, reducing sugars, and inorganic phosphorus contents. The three preparations showed no distinct differences in the contents of these matters (Table 1).

Table 1. Effect of time for crushing on the enzyme solution.

Crushing time	Inorganic phosphorus	Reducing sugar	Glycogen	Dry matter
sec.	mg. %	mg. %	mg. %	mg./ml.
30	26.2	11.4	19.5	18.0
60	26.1	12.2	18.3	18.4
180	26.9	10.8	21.8	18.6

Unit for analytical data, except for dry matter, means mg. per 100 ml. of the enzyme solution, and for all of the data in the following Tables and Figures this unit is adopted.

(ii) Effect of sedimentation

In order to determine the influence of cellular debris (particulate matter) in the muscle extract, by which the enzyme activity may be inhibited during the incubation at 37°C., two solutions were prepared. Whereas the one not-centrifuged contained particle matter, the other became free from such matter through the centrifugation.

Table 2 shows that the two enzyme preparations gave little difference in enzyme activity, and that the supernatant solution had an appreciable enzyme activity. Besides, the blend not centrifuged was undersirable for the reason of its high content of glycogen.

Table 2. Effect of sedimentation on the enzyme solution.

	Incubation time	Inorganic phosphorus	Reducing sugar	Glycogen
	hour	mg. %	mg. %	mg. %
Supernatant	0	26.2	11.4	19.5
	1	28.5	13.9	—
Supernatant + glycogen (200 mg.)	1	26.3	41.0	—
Blend	0	26.2	11.4	63.3
	1	29.9	19.3	—

Solution were incubated at 37°C.

(4) Determination of the enzyme activity

To 5 ml. of the enzyme preparation 1 ml. of 1% glycogen solution was added, and the mixture was incubated for 1 hour at 37°C. After the incubation, the reaction mixture was separated into two parts, of which the one was used for the determinations of reducing sugar and inorganic phosphorus after removing of protein with trichloracetic acid, and the other for the glycogen determination. To correct the interaction with the endogenous substances in the crude enzyme solution, a test without the addition of glycogen should also be performed.

(i) Estimation of inorganic phosphorus

Inorganic phosphorus was determined by the modification method of TAKAHASHI[6].

The sample solution which contained phosphorus below 90 γ was added to a 25 ml. volumetric flask which contains 2.2 ml. of 60% perchloric acid. After the dilution of the mixture to ca. 20 ml. with distilled water, 2 ml. of methol reagent (mixture of 1 g. of methol and 20 g. of $NaHSO_3$ in 100 ml. of distilled water) and 1 ml. of 8.3% ammonium molybdate solution were added. The flask was shaken strongly after the addition of distilled water so as to be 25 ml. Then the blue colour developed in 30~90 minutes was read by the use of Hilger Biochem Absorptiometer with its filter No. 70 put against the blank of distilled water, and the phosphorus content was calculated by means of a standard curve obtained for a pure KH_2PO_4 solution.

(ii) Estimation of reducing sugar

The method of HAGEDORN-JENSEN[6] was adopted in the present estimation of reducing sugar.

To the sample solution freed from proteinous matter by precipitation, 2 ml. of N/200 alkaline solution of potassium ferricyanide was added. The mixture was boiled for 15 minutes and cooled. To this were added 3 ml. KI-$ZnSO_4$ solution (12.5% of NaCl, 4.4% of KI, and 4% of $ZnSO_4 \cdot 7H_2O$) and 2 ml. of 5% acetic acid. Then the iodine liberated was titrated with N/200 $Na_2S_2O_3$, and reducing sugar was computed as glucose by the use of the calculation table.

(iii) Estimation of glycogen

To 1 ml. of the sample solution 1 ml. of 30% KOH was added, and the mixture was boiled for 30 minutes in a water bath, and cooled. To this solution 5~10 ml. of 90% ethanol was added, then the crude glycogen was precipitated. The precipitate separated from the supernatant fluid by centrifugation was redissolved in distilled water, and precipitated twice with alcohol. The glycogen solution prepared by dissolving the precipitate in distilled water, was hydrolyzed with 2 ml. of 2.2% HCl for 3 hours. The hydrolysate was made to 50 ml. with distilled water, and the glucose was determined by the method for reducing sugar mentioned above. The blank test, as usual, was made by the same procedure on the solution which contained no glycogen.

(iv) Determination of phosphorolysis

Phosphorolysis is one of the chemical reactions in animal tissue, i.e., glycogen+inorganic phosphorus$\rightleftarrows$glucose-1-phosphate, which is catalyzed by phosphorylase. On the contrary, glycolysis means, in general, a reaction in which

glycogen or glucose decomposes to substances of lower molecular weight liberating inorganic phosphorus.

In order to determine the activity of phosphorylase, the ability of glycogen synthesis from glucose-1-phosphate referring to the ratio of free inorganic phosphorus to ester phosphorus is measured usually[8]. However, the authors, using the crude enzyme solution of fish muscle, attempted to examine the phosphorolysis of the muscle of fish *post mortem* from the estimation of inorganic phosphorus, reducing sugar, and glycogen in the muscle extract.

II. Results

(1) *Effect of pH*

The observation was made on the enzyme solutions incubated for 1 hour at 37°C., with pH values varied from 2.3 to 9.9 with Sörensen's citrate, Michaelis' veronal, and Menzel's carbonate buffers[8].

Table 3 and Fig. 1 show that the decrease or increase of inorganic phosphrous occurring after incubation without glycogen addition depended obviously upon the pH values of muscle extracts. From these results it should be considered that the increase of inorganic phosphorus occurs mainly in an acidic pH-range of pH 3.0~5.0, while the decrease or a little increase appears in rather an alkaline one of pH 7.5~9.0.

On the basis of these result, the optimum pH of phosphorylase was measured in the presence of glucose added as the substrate. As shown in Fig. 2, the decrease of inorganic phosphorus was most remarkable at pH 6.4 at which the phosphorolysis by phosphorylase may occurs significantly resulting in the production of the glycogen-phosphorus compounds.

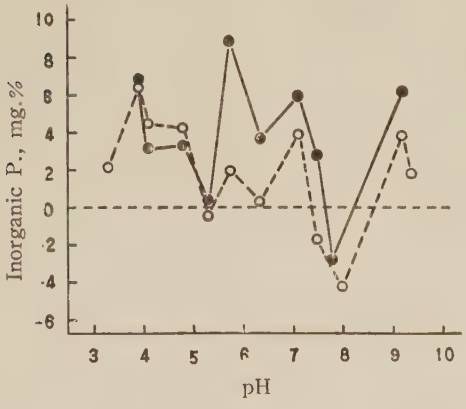


Fig. 1. Effect of pH on the variation of inorganic phosphorus in the enzyme solution during incubation. The enzyme solutions with various pH values were incubated for 1 hour at 37°C. without addition of substrate. The variation of inorganic phosphorus is expressed in mg. per 100 ml. of the solution. ●, sample 1; ○, sample 2.

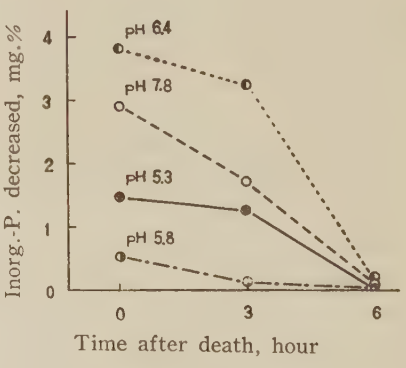


Fig. 2. Effect of pH on the consumption of inorganic phosphorus in the enzyme solution. The enzyme solutions prepared after 0, 3 and 6 hours from the time of death, were incubated for 1 hour at 37°C. with addition of glycogen (200 mg. to 100 ml. of the solution in concentration). The decrease of inorganic phsporus is expressed in mg. per 100 ml. of the enzyme solution.

Table 3. Effect of pH on the inorganic phosphorus contents in the enzyme solution.

Buffer solution		pH of enzyme solution		Inorganic phosphorus in enzyme solution					
Constitution	pH	Before incubation	After incubation	Before incubation		After incubation		A–B	
				1B*	2B*	1A	2A	1	2
				mg. %	mg. %	mg. %	mg. %	mg. %	mg. %
Citrate-HCl	2.34	3.29	3.10	—	20.2	28.8	22.3	—	2.1
	3.12	3.91	3.85	22.9	16.9	29.8	23.3	6.9	6.4
	3.45	4.10	4.20	31.5	18.6	34.7	23.0	3.2	4.4
	4.63	4.79	4.78	30.2	19.1	33.5	23.3	3.3	4.2
Citrate-HaOH	5.29	5.32	5.45	30.2	21.4	30.6	21.0	0.4	−0.4
	5.77	5.79	5.83	28.3	21.6	37.1	23.6	8.8	2.0
Veronal-HCl	6.63	6.35	6.40	32.7	24.9	36.4	25.2	3.7	0.3
	7.15	7.12	7.20	28.3	25.7	34.2	29.6	5.9	3.9
	7.52	7.49	7.55	28.8	31.9	31.6	30.3	2.8	−1.6
	8.22	7.83	7.90	33.3	—	30.6	—	−2.7	—
	8.50	8.00	8.05	—	35.5	—	31.3	—	−4.2
Carbonate-bicarbonate	9.47	9.19	9.20	24.9	22.8	31.1	26.6	6.2	3 8
	9.90	9.61	9.55	27.5	24.3	—	26.0	—	1.6

* 1 and 2 indicate the sample No. for two fishes.

The difference of optimum pH for the reaction which involvs inorganic phosphorus between these two experiments are not now evident, but it is suggested that the phosphorolysis at pH 6.4 in the experiment shown in Fig. 1 may be inhibited by the promotion of glycolysis against the reduction of the reaction at pH 7.5～8.0.

The results of the present experiment for the optimum pH of fish muscle phosphorylase are in close agreement with those of Cori[8] who measured the activity of the rabit muscle phosphorylease and found its optimum activity at pH 6.35.

The phosphorylase activity in fish muscle declined in proportion to the time after death, and as shown in a proceeding experiment, the enzyme activity after 6 hours from the time of death at 25°C. was perfectly destroyed.

(2) *Effect of time of enzyme preparation*

The experiment on the enzyme activity after the time of death of fish was carried out measuring the break-down of glycogen, the formation of reducing sugar, and the consumption of inorganic phosphorus. The result showed that the former two reactions proceeded considerably for the sample prepared after 1.5 hours from the time of death, but after 3 hours, the last stage of *rigor mortis*, the activity was lost rapidly as shown in Fig. 3. On the contrary, the change in inorganic phosphorus had an irregular form as shown in Fig 4. From these data, it is

suggested that the activity of phosphorolysis in fish muscle, at an early stage as after 1.5 hours from the time of death, is so stronger than the one of glycolysis immediately after the time of death, that the inorganic phosphorus decreases its content, while at the later stage as after 3 hours from the time of death increases rapidly.

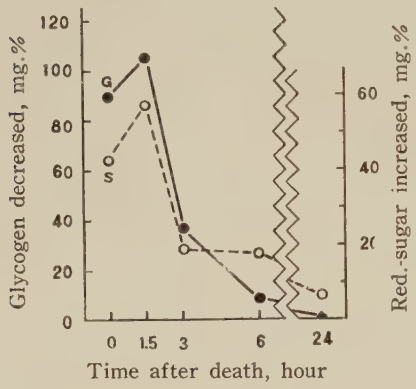


Fig. 3. Effect of preparation time on the breakdown of glycogen and the formation of reducing sugar. The enzyme solutions, prepared after 0, 1.5, 3, 6 and 24 hours from the time of death, were incubated for 1 hour at 37°C. with addition of glycogen. ●, glycogen; ○, reducing sugar.

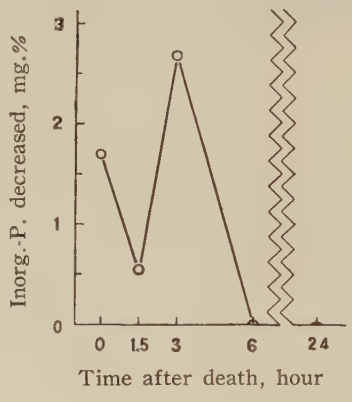


Fig. 4. Effect of preparation time of the consumption of inorganic phosphorus. Procedure was same with Fig. 3.

It was also found that the phosphorylase of muscle extract prepared after 1.5 hours from the time of death had more strong activity than that prepared immediately after the time of death (Fig. 3 and Fig. 4). From these interesting results it may be concluded that the enzyme at an early stage after the time of death of the fish is less soluble and has less activity, being combined with other organic substances such as protein, fat, carbohydrate, etc., but after 1.5～3 hours from the time of death the increase of the solubility and the activity of the enzyme may be appeared rapily by the breakdown of the enzyme compounds above mentioned.

(3) *Effect of temperature*

Temperature-effect on the activity of phosphorylase was apparently depended on a general role of enzyme action, namely, as shown in Figs. 5, 6 and 7, the lower temperature was given, the weaker activity was found.

However, the significant difference on the temperature-effect was caused by a freshness of fish flesh from which the enzyme solution was prepared. In the enzyme solution prepared immediately after the time of death, the activity of phosphorylase between 37°C. and 0°C. gave so clear difference that the ratio of the reaction velocity was indicated in 4:1. In the 4 hour incubation as shown in Figs. 5 and 6, the action of phosphorolysis of the enzyme solution at 0°C. was

already inhibited after 1 hour. On the other hand, the muscle extract prepared after 3 hours from the time of death had even the considerable phosphorylase activity at a lower temperature (0°C.), but the enzyme activity was accelerated so rapidly for a short time and was found to be inhibited in 30~60 minutes.

From the level for the disappearance of inorganic phosphorus shown in Fig. 7, it is evident that the phosphorylation occurred rapidly for the first period of

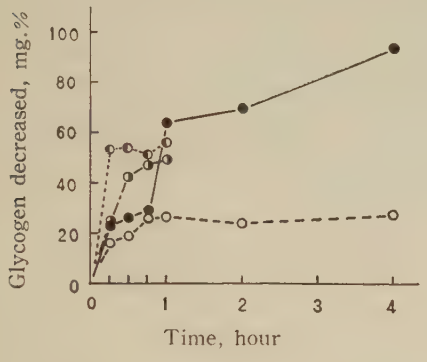


Fig. 5. Effect of temperature on the breakdown of glycogen. The enzyme solutions with glycogen were incubated for 4 hours at 0°C. and 37°C., and analyzed for the breakdown of glycogen at 1/4, 1/2, 3/4, 1, 2 and 4 hours after incubation. ●, the solution prepared immediately after the time of death and incubated at 37°C.; ○, at 0°C.; ◐, the solution prepared after 3 hours from the time of death and incubated at 37°C; ◑, at 0°C.

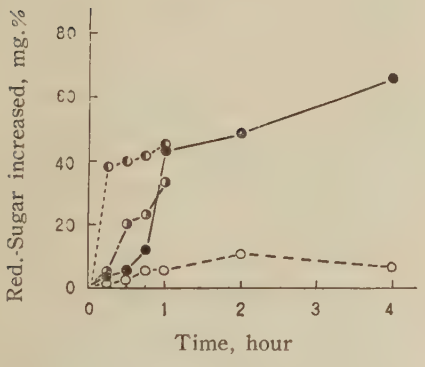


Fig. 6. Effect of temperature on the formation of reducing sugar. Procedure and indication are same with Fig. 5.

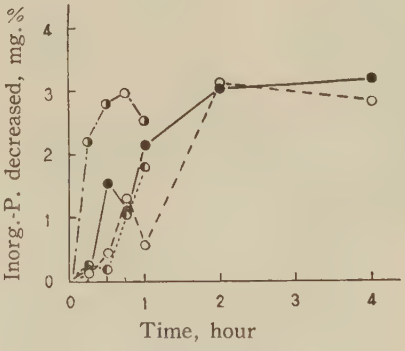


Fig. 7. Effect of temperature on the consumption of inorganic phosphorus. Procedure and indication are same with Figs. 5 and 6.

the incubation, but it began to go slowly and ceased after 2 hours, then the dephoshorylation might soon occur.

(4) *Effect of substrate concentration*

With the enzyme solution prepared from the most fresh sample, it was found that the breakdown of glycogen and the formation of reducing sugar were accelerated in proportion to the glycogen concentration added, but in the case of the less fresh sample, the special type of action which was inhibited rapidly in the glycogen concentration over 200 mg.%, while the normal increase of action was carried out till that concentration (Figs. 8 and 9).

Relation between the inorganic phosphorus and the glycogen concentration

had less accuracy to be explained for the irregular level or the inorganic phosphorus consumption as shown in Fig. 10. There is no obvious reason why the level of inorganic phosphorus in the use of the most fresh sample should be fallen

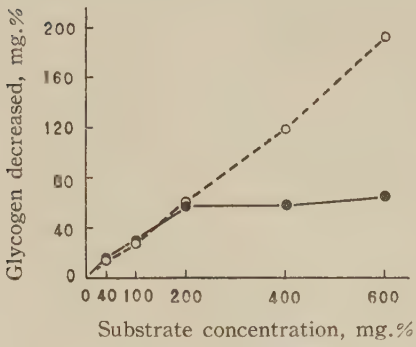


Fig. 8. Effect of substrate concentration on the breakdown of glycogen. The enzyme solutions were incubated for 1 hour at 37°C. with addition of glycogen in different concentrations (40, 100, 200, 400 and 600 mg. to 100 ml. of the solution). ○, the solution prepared immediately after the time of death; ●, the solution prepared after 3 hours from the time of death.

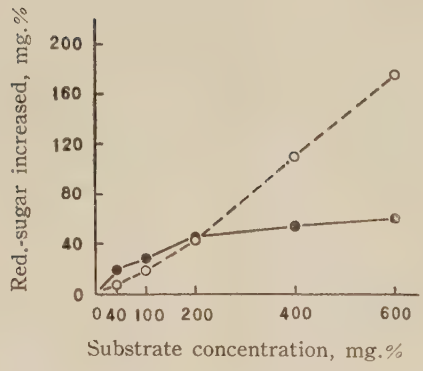


Fig. 9. Effect of substrate concentration on the formation of reducing sugar. Procedure and indication are same with Fig. 8.

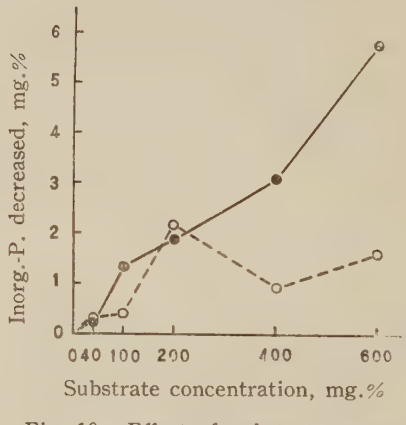


Fig. 10. Effect of substrate concentration on the consumption of inorganic phosphorous. Procedure and indication are same with Figs. 8 and 9.

at the lower glycogen concentration (200 mg.%) as at the higher concentration (400 or 600 mg.%). On the other hand, however, the disappearance of inorganic phosphorus, the phosphorolysis, in the use of the enzyme solution prepared from the less fresh muscle, proceeded approximately linearly corresponding to the glycogen concentration.

(5) *Enzyme activity of each part of fish body*

The phosphorylase activities of diffferent parts of fish were observed on the supernatant solutions prepared from the four parts of the body, i.e., anterior dorsal, posterior dorsal, ventral, and caudal muscles, respectively.

As shown in Figs. 11, 12 and 13, there was no evident difference on the enzyme activities among four parts, but it may be said that the dorsal part, in-

cluding anterior and posterior, gave a little stronger activity of the enzyme than other parts. In this experiment, it was also obvious that the freshness of the enzyme preparation gave a considerable influence on the phosphorylase action.

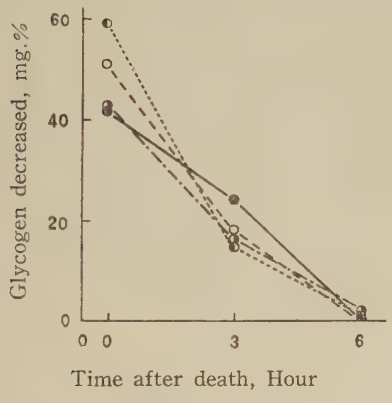


Fig. 11. Activity of the breakdown of glycogen in different parts of fish. The enzyme solutions were prepared from four different parts of fish after 0, 3 and 6 hours from the time of death, and incubated for 1 hour at 37°C. ●, posterior dorsal muscle; ○, anterior dorsal; ◐, caudal; ◑, ventral.

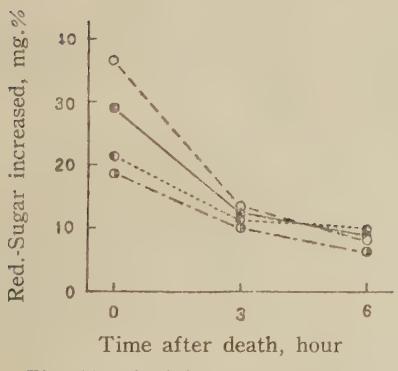


Fig. 12. Activity of the formation of reducing sugar in different parts of fish. Procedure and indication are same with Fig. 11.

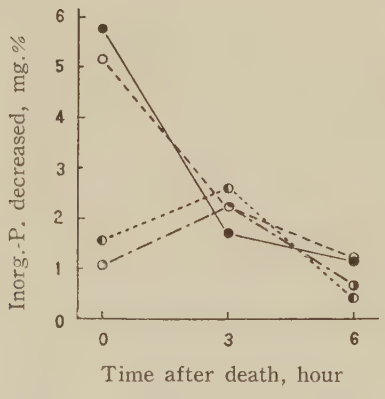


Fig. 13. Activity of the consumption of inorganic phosphorus in different parts of fish. Procedure and indication are same with Figs. 11 and 12.

Discussion

In the present experiment, the phosphorolysis of a marine fish muscle was investigated with the measure of the breakdown of glycogen, the formation of reducing sugar, and the release or the combination of inorganic phosphorus in the presence or absence of glycogen added as substrate.

In general, the enzyme activity, especially the enzymes as desmolase, of fish muscle developes rapidly its action at the beginning of the reaction, and after a short time it slows down suddenly by any inhibitance.

Such specificity of the enzyme of fish muscle seems to be due to the higher sensitivity of fish to temperature than the other animals by the fact that fish is cold-blooded, namely, the enzyme activity of fish muscle extract is greatly

influenced with its freshness. In the present experiment, therefore, the phosphorolysis is possible to be involved in the category mentioned above. These hypothesis would be proved by the following facts shown in our investigation.

The phosphorolysis of the supernatant solution from the sample, stored at 25°C. for 3 hours from the time of death, reached to a higher level for only 15 minutes at 37°C., and for 30 minutes at 0°C., while the phosphorylase solution from the most fresh sample rised its activity linearly for some time. The considerable loss of the enzyme activity in phosphorolysis for its temperature sensitivity or the freshness of fish may be mainly depended on the hydrolysis of phosphorylase by a certain protease, or on the presence of an inhibitor. Therefore, the market fish will be impossible to use as a sample for the enzyme preparation in view of the freshness. Although the problem with adenylic acid[8,10] which activates phosphorylase b (inactive form of the enzyme) was not clarified in this work, further investigation should be required.

Summary

(1) The phosphorylase in fresh muscle of a Japanese sea-bass was recognized to have its highest activity at approximately pH 6.4 as other mammals of which the optimum pH was found at 6.35, and the activity of the fish phosphorylase is more inhibitory in acidic side than in alkaline side.

(2) The phosphorylase of a sample stored at 25°C. lost its activity in proportion as the lapse of time and disappeared perfectly after 6 hours from the time of death.

(3) The freshness of fish had a great influence on the phosphorylase activity. The enzyme solution, prepared immediately after the kill of fish, activated linearly its action in the incubation at 37°C., although that obtained from a freshless sample represented a rapid reaction velocity and went to an end of the reaction after only 15 minutes.

(4) The phosphorylase contained in the most fresh muscle had only a lower level of activity at 0°C. in comparison with one at 37°C., but in the case of a freshless sample the activity at 0°C. as well as at 37°C. was in a higher level.

(5) A dorsal part of fish body was a slightly concentrated source of phosphorylase than other parts.

References

1) Yamada, K. and Simma, Y.: Bull. Japanese Soc. Sci. Fish., **14**, 23 (1948).
2) Yamada, K., Simma, Y. and Suzuki, T.: ibid., **14**, 41 (1948).
3) Yamada, K.: ibid., **14**, 44 (1948).
4) Amano, K., Bito, H. and Kawabata, S.: ibid., **19**, 487 (1953).
5) Fujimaki, M. and Kojo, K.: ibid., **19**, 499 (1953).
6) Takahashi, Y.: Med. and Biol., **26**, 235 (1953).
7) Hagedorn, H. C. and Jensen, B. N.: Biochem. Zs., **135**, 46 (1923), and ibid., **137**, 92 (1923).
8) Cori, G. T., Illingworth, B. and Keller, P. J.: in "Methods in Enzymology"

(COLOWICK, S. P. and KAPLAN, N. O., edt.), Volume 1, 200, Academic Press Inc., New York (1955).
9) Handbook of Biochemistry (in Japanese), 957, Gihodo Co. Ltd., Tokyo (1953).
10) HASSID, W. Z., DOUDOROFF, M. and BARKER, H. A.: in "The Enzymes" (SUMMER, J. B. and MYRBÄCK, K., edt.): Volume 1, Part 2, 1025, Academic Press Inc., New York (1951).

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

BIOCHEMICAL STUDIES ON THE VITAMIN A IN FISH VISCERA
IV. LIPOXIDASE IN LIVER AND DARK MUSCLE

Toyoki Ono, Fumio Nagayama and Tadanao Sasaki*

(Received Jan. 16, 1957)

Previous studies[1),2)] have shown that the enzyme in fish livers oxidizing vitamin A may be analogous to soybean lipoxidase.

In general, lipoxidase found in plant tissues has a function which produces a certain peroxide compound by the oxidation of unsaturated fatty acids, and if the carotenoids present there together, they ushold be secondarily oxidized. Therefore no direct oxidation of vitamin A will be occurred by lipoxidase in fish livers. Concerning the lipoxidase action of animal tissues, there have been some of the investigations in which these authors asserted that the lipoxidase action of animal tissues is not due to an enzyme system but is introduced by a heme-compound from a viewpoint of the heme-compound being capable of causing the oxidation of fatty acids[3),4),5)].

Reiser *et al.*[6)] investigated the relations between the heme-compoundand the lipoxidase in bacon adipose tissue and presented an evidence for the presence of a certain enzyme-like substance. From the dark muscle of herring, Kahn[7)] isolated a heat labile and proteinous compound free from Fe which oxidize fatty acids and postulated that its oxidation of linoleic acid agrees with the reaction by soybean lipoxidase. He illustrated that his "lipoxidase" exhibits a linoleic acid-oxidation as resulting in the production of a new peroxide-compound which shows a maximum absorption spectrum at 234 mμ.

This finding is very interesting corresponding with the previous work of Brocklesby *et al.*[8)] that proved vitamin A to be oxidized by the liver of salmon. It is an important investigation because of an enzyme system involved in fat-metabolism of fish that the absence of lipoxidase in white flesh (ordinary flesh muscle) of herring is in contrast with the fact that the dark muscle contained an enzyme oxidizing fatty acids.

An available determination of the lipoxidase activity adopts an absorption spectrum method measuring the maximum absorption spectrum at 234 mμ of the primary oxidation products of linoleic acid[9)], but this process needs a highly purified enzyme solution in order to aovid error for the absorption of ultraviolet light. Another is a colorimetric method based on the indirect (secondary) oxidation of carotenoids by an oxidation product of linoleate; descolorations of β-carotene[10),11)], bixin (pigment of Bixa orellana)[12)], and crocin (pigment of Gardenia jasminoides)[13)] are measured.

Present paper reports the oxidation of β-carotene and methyl linoleate by the liver and dark muscle extracts of pacific mackerel and frigate mackerel. The oxidation of β-carotene was measured by the Hilger Biochem Absorptiometer and the linoleate by the Beckman DU Spectrophotometer.

* Present address, Japan Export Frozen Marine Products Inspection Corporation, Kohan Building, 3 Kasumigaseki-3, Chiyodaku, Tokyo.

Experiments

A. Oxidation of β-carotene.

Materials and Method.

(1) *Materials.*

As a source of the enzyme the following materials were used.

(a). Fresh livers of market pacific mackerel (*Scomber japonicus*) landed at Numazu, July 1954.

(b). Livers and dark muscle of frigate mackerel (*Auxis tapeinosoma*) which were caught by us off the coast of Numazu, August, 1954.

To those materials which were ground finely three times through a grinder 10 volumes of cold acetone was added, and the dried powders were defatted with 3 volumes of low boiling petroleum ether and obtained there through a sieve of mesh 80 were stored at −25°C.

(2) *Preparation of enzyme solution.*

Acetone dried powders were suspended in the cold in addition of M/15 phosphate buffer solution which was added 5 volumes for liver powders and 3 volumes for dark muscles. The brownish supernatant was obtained through the centrifugation of the mixture.

(3) *Reagents.*

(a). Acetone solution of β-carotene. In 100 ml. of acetone 10∼15 mg. of a crystalline β-carotene was dissolved and stored in an ice box.

(b). Acetone solution of methyl linoleate. Linoleic acid was obtained from fatty acids of linseed oil by a fractional distillation and the ester was prepared by ordinary method. With pure acetone, 5 g. of methyl linoleate was dissolved to 25 ml.

(c) Basic Pb-acetate solution. In 80 ml. of distilled water was dissolved 20 g. of basic Pb-acetate.

(4) *Method.*

In a test tube of 20 ml.-volume, 1 ml. of phosphate buffer, 0.1 ml. of solution (b) and 0.1 ml. of solution (a) were placed, and to this mixture 1 ml. of enzyme solution was added and incubated at 37°C. for a certain time. After the shaking of mixture in addition of 0.3 ml. of solution (c) and 10 ml. of acetone, the supernatants were obtained centrifuging (1500× *g.*) the mixture. The β-carotene content of the supernatant was determined measuring the optical density in use of a colorimeter. The blank test in the absence of active enzyme was carried out to correct the errors from the oxidation of β-carotene by oxygen, the adsorption of β-carotene by protein, and the color substances eluted from the enzyme solution.

In order to use the Hilger Biochem Absorptiometer for the exact measurement of β-carotene oxidation, the experiment was carried on and gave the following results.

Each maximum absorption spectrum of two β-carotene solutions in concentration of 0.15 and 0.25 mg./100 ml. was found at the 470 mμ. (Filter No. 47) as shown in Fig. 1.

Optical density of β-carotene solution increased linearly to the concentration

of 0.27 mg./100 ml. Therefore, as shown in Fig. 2, the most suitable range for the measurement of oxidation should be carried on the range below 0.25 mg./100 ml. in β-carotene concentration and 0.4 in optical density.

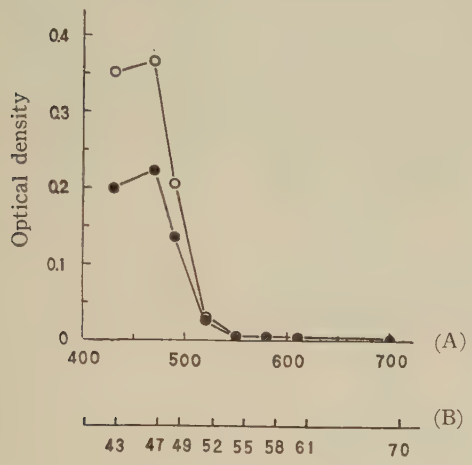


Fig. 1. Extinction coefficient at various wave lengths of aceton solution of β-carotene (Cuvett, 14 mm. depth), 0.246 mg.% (○), and 0.149 mg.% (●). (A), Wave length, mμ; (B), Filter No.

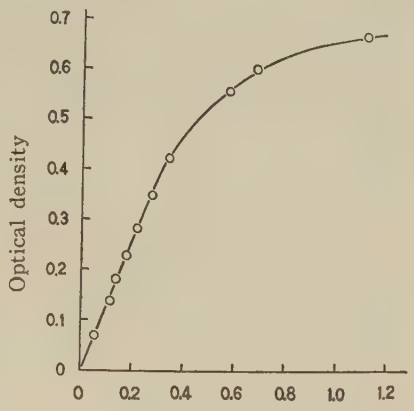


Fig. 2. Relationship between extinction coefficient and concentration of β-carotene. (Cuvett, 14 mm. depth).

Results and Discussion.

As shown in Fig. 3, the reaction velocity on oxidation of β-carotene by the fish tissue extracts was differed in accordance with the species of fish, their enzymatic actions of liver and dark muscle of frigate mackerel developed rapidly for 10 minutes in early stage but reached so onto an equilibrium, and the enzyme of dark muscle represented the activity as same as the one of liver.

On the contrary, the enzymatic oxidation of β-carotene by pacific mackerel liver extract increased so slowly the activity that the reaction had even no equilibrium after 60 minutes, and this enzyme activity increased further so slowly as the one of frigate mackerel. From these results, it is suggested that the oxidative reaction of enzyme may be rather attributed to the freshness of fish than the difference of fish species.

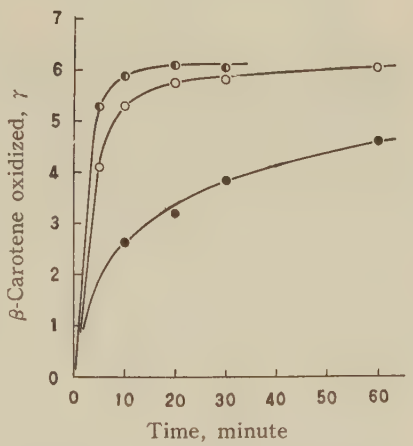


Fig. 3. β-Carotene oxidizing activities of the livers of pacific mackerel (●) and frigate mackerel (○), and of the dark muscle of frigate mackerel (◐). Reaction mixtures were incubated at 30°C. (pacific mackerel) and26° C. (frigate mackerel), pH 6.8. Amount of β-carotene oxidized by 1 ml. of an enzyme solution is expressed in γ.

(1) *Effect of pH upon the enzyme activity.*

β-carotene was used as substrate and the pH was adjusted to different values from 5.5~8.0 by phosphate buffer. The results indicate β-carotene (in γ) oxidized by 1 ml. of the enzyme solution after the incubation time in 10 minutes. The relative activities of the enzyme solution obtained from these sources at various pH values were shown in Fig. 4. It was observed that the enzyme was most active around pH 6.8 in each case of three experiments, but the activity falls much more quickly on the acid side of the pH value than the alkaline side.

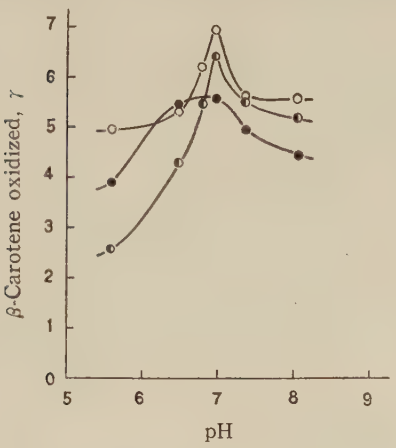


Fig. 4. Effect of pH on β-carotene oxidizing activities of the livers of pacific mackerel and frigate mackerel, and of the dark muscle of frigate mackerel. Reaction mixtures were incubated for 10 minutes at 26°C. for pacific mackerel and 20°C. for frigate mackerel. All of indication and unit are same with Fig. 3.

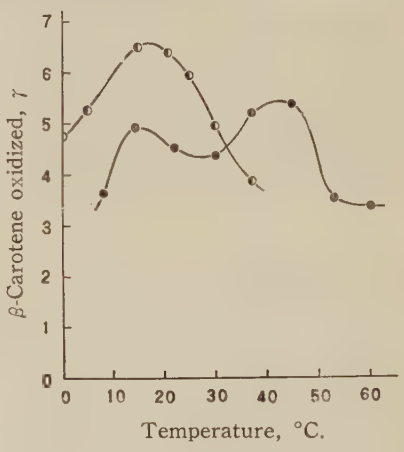


Fig. 5. Effect of temperature on β-carotene oxidizing activities of the liver of pacific mackerel and the dark muscle of frigate mackerel. Reaction mixtures were incubated for 10 minutes at pH 6.8. All of indication and unit are same with Figs. 3 and 4.

(2) *Effect of temperature on the enzyme activity.*

A series of trials was made at each of several temperatures, using equal quantities of enzyme together with an optimum amount of activator. The results are shown in Fig. 5.

The enzyme preparation from dark muscle of frigate mackerel exhibited the most strong activity at 15°C., but a rapid decrease above 25°C. However, the enzyme from pacific mackerel liver represented two peaks in activity at 15°C. and 45°C. Such a specificity on temperature-influence may have not ever been found in an enzyme of any other body tissues. It must be pointed out that such a specific type on reaction is not derived from the only one enzyme system, but the another enzyme or enzyme-like substance will be activated at 45°C.

(3) *Effect of fatty acids and hemin on oxidation of β-carotene.*

The enzymatic oxidation of β-carotene needs essentially the presence of unsaturated fatty acids from which the oxidation products are made by the enzyme action, and these compounds oxidize β-carotene secondarily.

The evidences for the oxidation of unsaturated fatty acid by hemin have been given by some investigators who observed that hemin has also an enzyme action as same as soybean lipoxidase. In the present experiments the authors proved that no oxidation of β-carotene was taken place in the absence of unsaturated fatty acids in even the addition of an amount of enzyme or hemin, against linoleic acid accelerated the natural activity of the enzyme and was caused in the rapid increase of the oxidation of β-carotene as shown in Table 1.

Table 1. Effects of fatty acids and hemin on the oxidation of β-carotene. The enzyme solution of pacific mackerel liver, 10% aceton solutions of linoleic or caprylic acids, 10 mg% hemin solution were used. Incubation time was 30 min. at 37°C., pH 6.8.

Linoleic acid solution	Caprylic acid solution	Hemin solution	Enzyme solution	β-Carotene Oxidized
ml.	ml.	ml.	ml.	γ
—	—	—	—	0
—	—	—	1.0	0
—	—	0.05	—	0
—	—	0.05	1.0	0
0.1	—	—	—	1.3
0.1	—	—	1.0	3.4
0.1	—	0.05	—	4.0
0.1	—	0.05	1.0	4.2
—	0.1	—	—	0.3
—	0.1	—	1.0	1.1
—	0.1	0.05	—	0.6
—	0.1	0.05	1.0	1.6

In the presence of a saturated fatty acid such as caprylic acid in stead of linoleic acid, the oxidation of β-carotene was exhibited in some rates, although it did not increase activity so much as in the case of linoleic acid. However, there is a doubt in this result whether the caprylic acid is a precursor of peroxide compound to oxidize β-carotene or the oxidation of β-carotene is attributed to the impurities in caprylic acid.

There is no evidence in the present experiments whether the enzymatic oxidation of β-carotene will be caused by an oxidase, lipoxidase or by an active compound, hemin.

B. Oxidation of linoleate.

Material and Method.

(1) *Material.*

The fresh livers of pacific mackerel which were landed at Numazu were used. To livers ground finely three times through a grinder, 10 volumes of cold acetone was added, and they were treated with same process as experiment 1.

Enzyme solution was prepared from the liver acetone powder being extracted with 5 volumes of M/15 phosphate buffer, pH 6.8.

(2) *Substrate.*

(a) Methyl linoleate. Methyl linoleate was isolated from the mixed fatty acid methyl esters of linseed oil through a fractional distillation in usual manner.

(b) Substrate emulsion. The mixture of 30 ml. of M/15 phosphate buffer, pH 6.8, and 0.3 g. of emulsifyer, CMC (carboxy methyl cellulose), placed in a Ehrenmyer flask of 200 ml. volume, was emulsifyed in vacuum using a magnetic stirrer in additon of methyl linoleate.

(3) *Method.*

Each 1 ml. of substrate emulsion and enzyme solution was placed in a test tube with a stopper. After the incubation of the mixture at 37°C. for a certain time, 10 ml. of ether was added and the methyl linoleate was translated in an ether layer by a vigorous shake. From this ether solution, 1 ml. was taken in a volumetric flask and diluted with pure alcohol to a volume of 100 ml. or more in a concentration suitable for measurement of its absorption with a Beckman spectrophotometer. At the same time methyl linoleate content was measured using 1 ml. of the ether solution of methyl linoleate.

Results and Discussion.

The results from the experiments of enzymatic oxidation of linoleate by pacific mackerel liver extract were shown in Figs. 6 and 7. The absorption

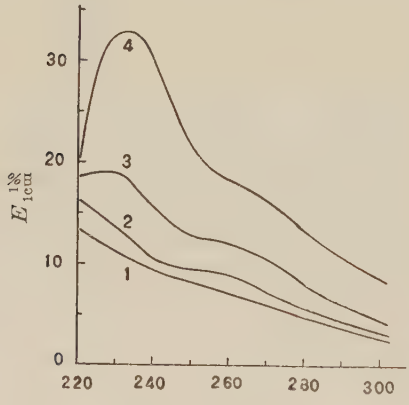


Fig. 6. Absorption curves of methyl linoleate in ethanol. Curves indicate methyl linoleate incubated for 10 minutes (1), 20 minutes (2), 30 minutes (3), and 60 minutes (4), with mackerel liver enzyme at 30°C., pH 6.8.

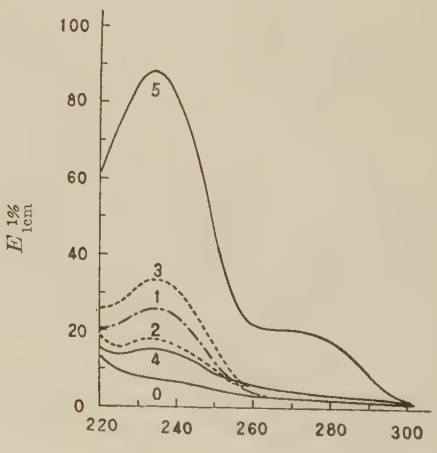


Fig. 7. Absorption curves of methyl linoleate in ethanol. Curves indicate methyl linoleate of original (0), aerated for 5 hours at 50°C. (1), incubated for 20 hours at 26°C., pH 6.8 in emulsion with mackerel liver enzyme (2) and without enzyme (3), and incubated for 40 hours at 26°C. with the enzyme (4) and without enzyme (5).

spectra in an early stage of reaction, after 10 and 20 minutes from the time of reaction were only illustrated as same as the non-oxidized substrate on which absorption spectrum was shown as curve No. 0 in Fig. 7. However, after 30

minutes the activity of liver extract was appeared so suddenly that the oxidation products represented a results near the typical optical density of a oxidation product linoleate of which the absorption spectrum has been found to have a maximum at 234 mμ, and being proceeded the enzyme activity appeared so strongly that typical type of linoleate oxidation was exhibited in a absorption spectrum.

Effects of long duration of the oxidation on the spectra were studied resulting in a interesting observation. The optical density of oxidation products obtained by a long time oxidation was so much dropped compared with the one of 1 hour-oxidation, and the peak at 234 mμ has been no more appeared in the incubation at 26°C., for 20~40 hours (curves Nos. 2 and 4 in Fig. 7).

On the contrary, in a series of blank tests using no enzyme the auto-oxidations of linoleate occurred almost normally and the oxidation products gave each maximum absorption spectrum, especially occurring distinctly in an oxidation-test at 26°C., for 40 hours (curve No. 5 in Fig. 7).

It is suggested that the peroxide compound mentioned above would be broken down by an enzyme-like substance in liver, being less unstable to heat or oxidation, in stead of the denaturation of lipoxidase that involvs the inactivity for the oxidation of linoleate oxidation by soybean lipoxidase.

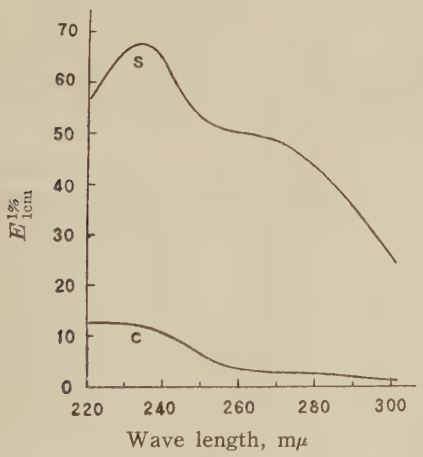


Fig. 8. Absorption curves of methyl linoleate in ethanol. Curves indicate methyl linoleate incubated for 2 hours at 30°C., pH 6.5 with soybean lipoxidase (S) and without enzyme (C).

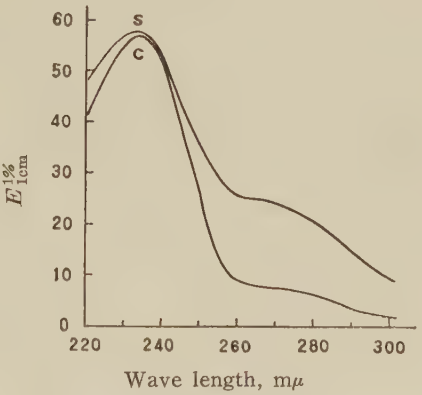


Fig. 9. Absorption curves of methyl linoleate in ethanol. Curves indicate methyl linoleate incubated for 18 hours at 30°C., pH 6.5 with soybean lipoxidase (S) and without enzyme (C).

The measurement of the activity of soybean lipoxidase was carried on the oxidation of substrate in same manner as the pacific mackerel liver preparations, but the absorption spectrum of the oxidation product from substrate was not identical to the result shown in the Khan's experiments with herring dark muscle in which he found two maxima of absorption spectrum at 234 and 270 mμ, but our experiments exhibited only one maximum as same as the liver extracts except the slightest rising of absorption at near 270 mμ (Fig. 8).

The oxidation by long duration by soybean lipoxidase did result in not samely to the experiments of fish liver as in Fig. 9, namely both measurements after 2 and 18 hours of the incubation at 30°C. exhibited same absorption spectra, possesing a maximum at 234 mμ.

A suggestion will be made from that the soybean lipoxidase may be to say to be more less unstable than the one of liver extracts.

From the present experiments it is not yet distinct whether the oxidation mechanism by a liver enzyme preparation is identical to the one of soybean lipoxidase or not.

It is to say that the oxidation of linoleate by fish liver or dark muscle extracts is carried by a lipoxidase but by hemin. Some of authors making these investigations asserted the absence of lipoxidase in animal tissues. However, Khan proved in herring dark muscle the presence of lipoxidase and unknown activator which were isolated in concentration free from hemin and oxidized linoleic acid.

Summary

(1) β-Carotene was easily oxidized by the fish liver and dark muscle extracts in a presence of linoleic acid and the oxidation was attributed to a lipoxidase like enzyme contained in fish tissuses.

(2) The liver and dark muscle of frigate mackerel were proved to have a high enzyme activity on oxidation of β-carotene which rapidly increased, while the pacific mackerel liver did not so strongly exhibit in an enzyme activity as the formers.

(3) Each enzyme prepared from fish tissues represented the maximum activity at pH 6.8 and was inactivated more rapidly in the acid side than the alkaline side.

(4) Optimum temperature of lipoxidase-like enzyme was measured at 15°C. in the case of dark muscle of frigate mackerel, but the pacific mackerel liver extracts had two optimum temperatures at 15°C. and 45°C.

(5) The enzymatic oxidation of linoleate by pacific mackerel liver extract may be occurred in near after 30 minutes from the time of incubation at 30°C., pH 6.8, and was obviously represented the typical oxidation type after 60 minutes, which was also illustrated in the autoxidation.

(6) Although the enzymatic oxidation should be carried out for long time at 30°C., 18 hours or at 26°C., 40 hours, soybean lipoxidase was not almost inactivated, while the enzyme from fish tissues was rapidly destroyed its activity. The optical density in the experiment with soybean lipoxidase indicates further so high value.

References

1) Ono, T. and Nagayama, F., J. Tokyo Univ. Fish., **39**, 1 (1952).
2) Ono, T. and Nagayama, F., ibid., **41**, 153 (1955).
3) Watts, B. M. and DaHwei Peng, J. Biol. Chem., **170**, 441 (1947).
4) Tappel, A. L., Food Res., **17**, 550 (1952).
5) Tappel, A. L., ibid., **18**, 104 (1953).

6) Reiser, R., J. Am. Oil Chem. Soc., **26**, 116 (1949).
7) Khan, M. M. R., J. Fish. Res. Bd. Canada, **9**, 393 (1952).
8) Brocklesby, H. N. and Rogers, N. I., Fish. Res. Bd. Canada, Prog. Rep. Pac., 50, 4 (1941), and Ind. Memorandum, No. 6 (1941).
9) Theorell, H., Bergström, S., and Åkeson, Å., Pharm. Acta Helv., **21**, 318 (1946).
10) Reiser, R. and Fraps, G. S., J. Assoc. Off. Agr. Chemists, **26**, 186 (1943).
11) Balls, A. K., Axelrod, B. and Kies, M. W., J. Biol. Chem., **149**, 491 (1943).
12) Smith, G. N. and Sumner, J. B., Arch. Biochem., **17**, 75 (1948).
13) Fukuba, H., J. Agr. Chem. Soc. Japan, **25**, 483 (1952).

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

FURTHER OBSERVATIONS ON THE CAUSATIVE BACTERIA OF THE EPIDEMIC DISEASE LIKE FURUNCULOSIS OF RAINBOW-TROUT

Toshikazu Hoshina

(Received Jan. 16, 1957)

The writer published a previous report dealing with a epidemic disease of rainbow-trout in this journal (Vol. 42, No. 1, 1956). For this times, the author wishes to give a more complete description of the causative bacterium. The expence of this study was defrayed by the Grant in Aid for Experimental Scientific Research of the Ministry of Education, to which the writer offers his cordial thanks.

1 Strains

Following five strains were used for the study. All the strains were isolated from the rainbow-trouts (*Salmo gairdnerii*) which showed typical sign of the disease.

Strain 1—Isolated on 21, Feb. '53, from the kidney of a fish originated from the pond of the Gakunan Trout Cultural Co., Ltd. in Shizuoka Pref..

Strain 2—Isolated on 8, April '54, from the lession in musculature of a fish obtained at the Hakojima Trout Hachery in Gumma Pref..

Strain 3—Isolated on 10, Nov. '54, from the kidney of a fish which came from the Taissen Trout Hachery of Tottori Pref..

Strain 4—Isolated on 13, Dec. '55, from the Kidney of a fish collected at the Mazuda Trout Cultural Farm in Tottori Pref..

Strain 5—Isolated on, 4 May '56 from the lession in musculature of a fish collected at the Ayabe Fish Cultural Farm at the vicinity of the Mishima City of Shizuoka Pref..

2. Morphological and Cultural Character

Morphology: Slightly curved bacilli, with somewhat pointed or rounded ends, occurring singly, $1.1\sim3.6\mu\times0.35\sim0.6\mu$ in size. Motile, possessing a polar flagellum. Non-sporing and non-encapsulated. Gram-negative.

Agar plate culture: Small colonies 0.5~2 mm in diameter, gray-whitish, smooth, moist and transparent. They present dark purple colour within one minutes by 1% aqueous solution of paraphenylenediamine.

Agar slant: Very slightly grayish brown, smooth, glossy, semitransparent, moist and slimy surface growth. Condensed water whitish-trubid, which is slimy.

Blood-agar plate: The medium was prepared by adding about one part of

carp-blood into nine parts of the agar. The colonies are circular with a smooth convex surface, glistening, slightly dark grayish black, intransparent, showing a zone of hemolysis around them in two or three days at 23°C.

Endo's medium: Small reddish colonies appeared in one day at 23°C.

Gelatin stab: Very slowly napiform or stratiform liquefaction. The author reported that the bacterium does not liquefy gelatin in the previous report[4]. However, it should be recognized that the bacterium is possessing a nature to liquefy it.

Potato: Lustrous, moist and pale yellowish-brown growth in two days at 22~23°C. Afterward the growth and medium becoming brownish.

Milk: All the strains produced a soft clot in three to eight days at 22–23°C. Though acidic at first, the media showed basic reaction afterward and peptonized.

Broth: Scanty growth, uniformly turbid, grayish-white pellicle and sediment.

Actions for carbohydrates: All the strains showed the same actions as follows: Acid but not gas from arabinose, dextrose, fructose, galactose, mannose, maltose, trehalose, dextrin, glycogen, starch and mannit. No acid and no gas from rhamnose, xylose, cellubiose, lactose, saccharose, inulin, salcin, dulcit, adonit, glycerin, inosit, sorbit, and sorbose.

Biochemical characteristics: The experimental results are compiled in the Table 1.

Table 1. Biochemical characteristics.

Items \ Strains	1	2	3	4	5
H_2S–Formation	–	+ (very weak)	+ (very weak)	±	–
Indol–Formation	–	+ (very weak)	+ (very weak)	–	–
Hydrolysis of starch	+	+	+	+	+
Methyl red test	–	–	–	–	–
Voges-Proskauer's reaction	–	–	–	–	–
Catalase reaction	+	+	+	+	+
Formation of nitrites from nitrates	+	+	+	+	+
Reduction of litmus	–	–	–	–	–
Reduction of methylen blue	+	+	+	+	+

Relation to air, temperature and PH: Aerobic. Optimum temperature 15~20°C, but growth occurs at 5~30°C. No growth at 37°C. Optimum pH 7.4; the growth is possible at the range 6.3 to 9.7.

As above-described, all the strains showed almost the same characteristics.

3. Nutritive requirement

The Arima's synthetic medium (p. 290[1]) was used for the study, but the writer modified it as it contains 5 g of glucose and 5 g of Na-citrate per liter. The media lacking one kind by one kind of amino acids or vitamins were prepared according to the formula and the bacterium was cultured successively in these media at 23~24 C°. At first, the agar slant cultured bacteria which suspended in sterilized physiological salt solution were transplanted into the media. The results are shown in the Tables 2 and 3.

The Tables 2 and 3 show the results in the case of the strain 2. The other four strains also showed about the same characters. It was observed that some strains do not grow in 24 hours at the start of the cultures but the growth

Table 2. The result of the successive cultures in the synthetic media deficient in one kind of amino acid.

* The listed amino acid was excluded from the medium.

Medium*	Growth of each generation				
	1	2	3	4	5
Standard medium	+	++	++	++	++
Ala.	+	++	++	++	++
Asp.	+	+	+	+	+
Glu.	+	+	+	+	+
Arg.	+	+	+	+	+
Lys.	+	++	++	++	++
His.	+	++	++	++	++
Ileu.	+	+	+	+	+
Met.	+	+	+	+	+
Phe.	+	+	+	+	+
Pro.	+		+	+	+
Thr.	+	+	+		
Tyr.	+	++	++	++	++
Val.	+	+	+	+	
Try.	+	++	++	++	++
Cys.	+	++	++	++	+
Ser.	+	++	++	++	++
Gly.	+	++	++	++	++
Leu.		±	−	−	−

Table 3. The result of the successive cultures in the media free from one kind of vitamin.
* The listed vitamin was excluded from the medium. The media were prepared by using vitamin free casa amino acid "Defco".

Medium*	Growth of each generation				
	1	2	3	4	5
Standard medium	+	+	+	+	+
Free from all vitamins	+	+	+	+	+
Vitamin B_2	+	+	+	+	+
Vitamin B_1	+	+	+	+	+
p-Aminobenzoic acid	+	+	+	+	+
Pyridoxine-HCl	+	+	+	+	+
Pyridoxal	+	+	+	+	+
Ca-dl-Pantothenate	+	+	+	+	+
Nicotinic acid	+	+	+	+	+
Biotin	+	+	+	+	+
Folic acid	+	+	+	+	+

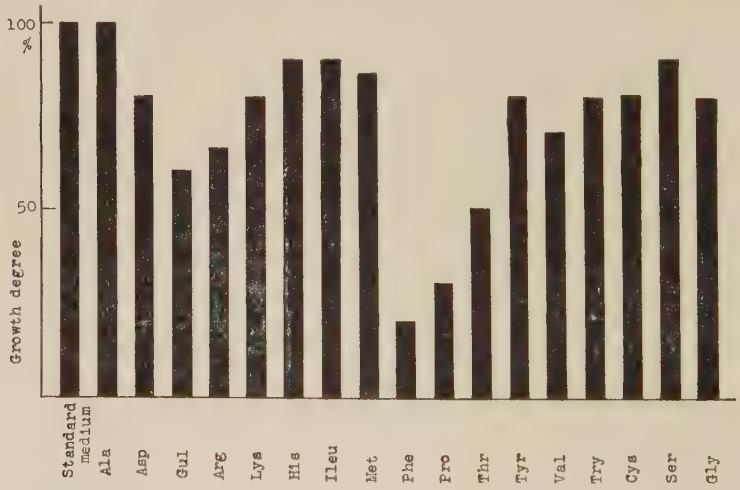


Fig. 1. Showing the growth degree of the bacterium in each medium, from which one kind of amino acid was exculded. It was measured by using photo-electro-colorimeter, and calculated on the basis of the growth in basal medium which determined as 100%. Filter: 660. Cultured in 48 hours at 23°C.

was seen within 48 hours. All the strains did not grow successively in the medium not contained leucine. Therefore, leucine is recognised as an essential

amino acid for the growth of the bacterium. However, in the case of the strains 1, 3 and 4, the very scanty growth was observed in the medium lacking leucine after 48 hours. As the Fig. 1 shows, the growth was influenced considerably by the lack of gultamic acid, arginine, phenylalanie, prolin, and threonine, and the other amino acids did not produced sever effect upon the growth.

The successive cultures in the media free from each vitamin were possible in the cases of the all strains, however the growth in the medium free from all vitamins was inferior about 20% than that of in the basal medium. Riboflavin had no effect on the growth. The absence of thiamine or p-aminobenzoic acid influenced more or less on it (Fig. 2).

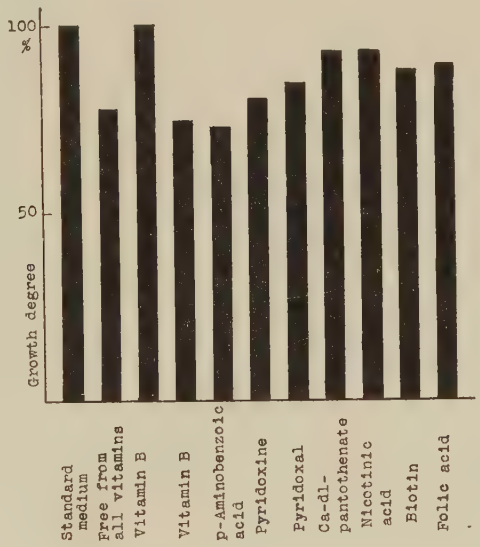


Fig. 2. Showing the growth degree of the bacterium in the medium free from one kind of vitamin. Cultured in 48 hours at 23°C. The measurements and calculations are same as the preceding experiment.

At any rate, it should be recognize that the present bacterium does not require any vitamins for it's growth in the medium.

4. Some biological characters

The experiments were carried out by using the strain 1, 3 and 5. Thermal death point: Killed in 10 minutes at 40°C; 5 minutes at 43°C 2.5 minutes at 45°C and 1 minute at 48°C. (The agar slant cultured bacteria in 24 hours at 23 ~24°C were tested).

Resistance against some disinfectants: The experimental results are compiled in the Table 4.

Resistance against sulfa drugs and antibiotics: The growth was observed by inoculating the tentative bacteria in 10 cc bouillon containing sulfa drugs or antibiotics. The results are shown in the Tables 5 and 6.

As the Table 5 shows, thiasin exhibited the strongest bacteriostatic power against the bacterium.

Table 4. Resistance against disinfectants.

Remark: One normal loop of the bacteria collected from the agar slant culture in 24 hours at 23～24°C was steeped in 10 cc of the disinfectants.

Disinfectant	Concentration	Lethal time (minutes)
Chlorine	1: 300,000	2.5
	1: 400,000	5
	1: 500,000	20
$KMnO_4$	1: 10,000	2.5
	1: 20,000	7.5
	1: 30,000	10
$Ca(OH)_2$	1: 1,000	2.5
	1: 2,000	5
	1: 3,000	15
	1: 5,000	2
Malachite green	1: 35,000	5
	1: 40,000	10
	1: 50,000	25
	1: 80,000	40
	1: 100,000	60

Table 5. Resistance against sulfa drugs.

* Used as Na—salt. ** 6-sulfanylamide-2, 4-dimethyl pyrimidine.

mg in 10 cc Bouillon / *Sulfa drugs	0	1.0	2.5	5.0	10
Control	##				
Sulfamine		+	+	±	–
Sulfadiazine		+	+	±	–
Thiasin		+	–	–	–
Sulfaziazol		##	##	##	+
**Domian		+	+	±	–

Pathogenicity for frog: The experiment was carried out in summer season (Sept. 1, '55) and two strains (No. 2 and 3) were tested. Frogs (*Rana nigromaculata nigromaculata* (HALLOWELL) varying 10～20 g in body weight divided into two groups consisting of three heads were inoculated intramuscularly of leg with 0.25 c.c. suspension of the bacteria (added about 4 mg of the bacteria collected from the agar slant culture in 24 hours at 22°C in 10 cc physiolosical salt solution). All the animals were survived without any sign of abnormality (continu-

Table 6. Resistance against antibiotics.
Results obtained from the cultures in 24 hours at 23–24°C.

Antibiotics	The concentration arrested the growth completelly (mg/10 cc bouillon)
Teramycin	0.005
Aureomycin	0.002
Streptomycin	0.1
Chloromycetin	0.005
Penicilin G	800 units
Bacitracin	800 units

ed the observation one week).

5. Remarks

There are two species of the genus *Vibrio* which are pathogenic for fish—*V. anguillarum* Bergmann 1909 and *V. piscum* David, 1927. Of these *V. piscum* is a species in Austria, isolated from the diseased carps caused by the bacterium. David stated that the species dose not attack trouts in natural conditions. On the other hand the writer's species is a vibrio attacking rainbow-trout and man can not find a spontaneous disease of carps caused by it. However, *Plecoglossus altivelis* and *Moroco steindachneri* are attacked by it and the pathological anatomical findings of the diseased fishes resemble to that of in the case of rainbow-trout.

Now as compared with the two species the differences follow:

Items	*V. Piscum*	Present species
Morphology	Slightly curved bacilli with a polar flagellum, $2\mu \times 0.3 \sim 0.5\mu$. Gram-negative	Do., $1.1 \sim 3.6\mu \times 0.35 \sim 0.6\mu$. Gram-negative
Agar plate culture	Colonies circular, yellowish, glistening, smooth, entire	Colonies circular, grayish white, moist, transparent, smooth, entire
Agar slant	Pale yellowish, flat, transparent growth. Condensed water clear with sediment	Smooth, glestining, transparent, moist and slimy growth. Condensed water whitish trubid with sediment
Endo's medium	At first colonies colourless, afterward becoming reddish in the center	Reddish colonies occur in 24 hours
Gelatin stab	Napiform liquefaction. Fluid clear	Very slowly napiform or stratiform liquefaction. Fluid clear

(Continued)

Items	*V. Piscum*	Present species
Potato	At first flesh-coloured growth, afterward becoming reddish chocalote-colour	Glistening, smooth, light yellowish growth. Afterward becoming brownish
Broth	Slight turbidity with thin pellicle	〃
Milk	Soft coagulum, peptonized, alkaline	〃
Production of H_2S	Positive (strong)	Positive (very weak) or negative
Indol-Formation	Positive	Positive (very weak) or negative
Production of gas from carbohydrates	None	〃
Formation of nitrites from nitrates	Negative	Positive

As described above the differences in biological characters between the two species are not to consider them identical. It seems that it is adequate to rank the present form under *Vibrio piscum*. The writer recognizes it a variant form of *V. piscum* and names *V. piscum var. japonicus* n. var.

Summary

The results of a further study of the causative bacterium of epidemic disease like furunculosis of rainbow-trout are presented.

The bacterial species is proposed as a new variant form of *V. picum*, which is named *V. piscum var. japonicus* n. var.

Concerning to the nutrative requirements of the bacterium, it is recognized that leucine is essential amino acid for it's growth and vitamins are not required in the Arima's synthetic medium.

The experimental results concerned with the resistance of the present bacterium against some disinfectants, sulfa drugs and antibiotics are compiled in the Tables 4, 5 and 6.

Literature

1) AKABORI, S. and MIZUSHIMA, S. 1954. Chemistry of protein (in Japanese). Vol. 1 Kyoritsushippan, Tokyo.
2) BERGMANN, A. M. 1909. Die rote Beulenkrankheit des Aals. Berichte Biol, Versstation in München. Bd. 2 S. 10～54.
3) DAVID, HANS. 1927. Ueber eine durch choleraähnliche Vibrionen hervorgerufene Fischseuche. Bakt. u. Parasitk. Bd. 1 Abt. Orig. 102, S. 46–60.
4) HOSHINA, T. 1956. An epidemic disease affecting rainbow-trout in Japan. Jour. Tokyo Univ. Fish. Vol. 42, p. 15～16.
5) PHILIP, G. J. 1952. A rapid presumptive test for furunculosis in fish. Prog. Fish-Cult. Vol. 14, p. 74～75
6) ROBERT, S. B. et al. 1948. Bergey's Manual of determinative bacteriology. Sixth edition. Williams & Baltimore.

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

ON A TRYPANOSOME OF EEL

Toshikazu Hoshina and Tokuo Sano

(Received Jan. 25, 1957)

Working on the haematological study of eel, the senior author found a trypanosome in the blood of the fishes.

So far as the occurrence of the parasite in Japanese eel (*Anguilla japanica*) concerned, no report has been published until now, so we wish here to note the observation of the parasite.

We acknowledge to Prof. Densaburo Inaba of the Tokyo University of Fisheries and Mr. Yujiro Kubota, the Director of the Haibara Fish Cultural Fisheries Corperative Association in Shizuoka Pref. for their aids by supplying the necessary materials.

1. Material and Method

The healthy and diseased eels (suffering from Hireakabyo*) which obtained in August, 1956 from the eel-culture ponds at Yoshida district in Shizuoka Pref. were used for the study.

For the microscopic observations, the regular preparations stained with May-Giemsa's method were prepared with the blood collected from the heart.

2. Characteristics of the Trypanosomes

Of the thirty healthy eels, varying in weight 203.5 to 369.0 g, seven, and, of the fifteen diseased eels (133–232 g) three were infected by this trypanosome which presenting following characters.

The body is slender, with a comparatively long posterior flagellum and pointed anterior end, and 18 to 32 μ long by 1.2 to 2.5 μ wide. The blepharoplast is oval in form, lying at the anterior end of the body, and measuring 0.7~1.0 μ by 0.5~0.8 μ in size. The undulating membrane is well distinguishable from the body proper; the staining reaction is acidphillous. The nucleus is located at about the middle part of the body, exhibiting compact chromatin net-works, and 2.2~3.3 μ by 0.7~1.0 μ in size. The cytoplasm is structureless, presenting neutrophillous nature, but some individuals showed vacuolated figures. The flagellum is 22 to 49 μ long. The distance from blepharoplast to the anterior end of the nucleus is 9.5~18.0 μ. The ratio of the distance to the blepharoplast from the nucleus to the body length is 0.34~0.68.

* A bacterial disease of eel which is identical with the "Süsswasseraalrotseuche" in Germany.

The Fig. 1 shows the general configuration of the parasite.

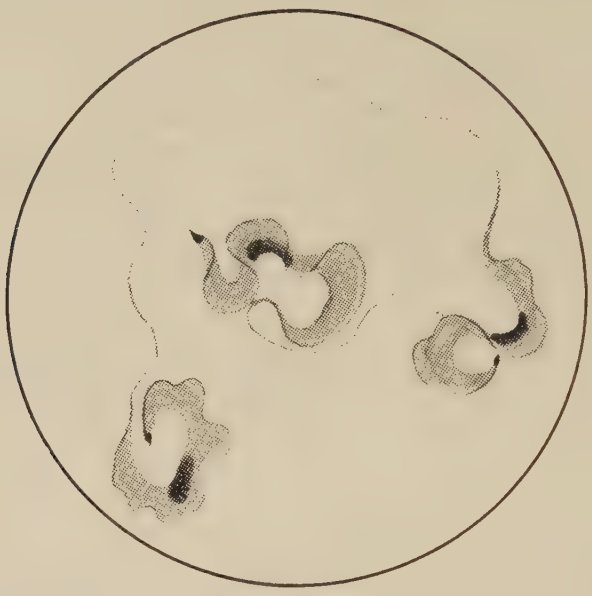


Fig. 1. The trypanosome found in the blood of *Anguilla japonica*. Stained with May-Giemsa. ×1200. Tokuo SANO *del*.

3. Remarks

There are 8 known species belonging to the trypanosomes of fresh water fishes; of these *Trypanosoma granulosum* LAVERAN et MESNIL occurs in eel (*Anguilla vulgaris*). Comparison of features and dimensions of between *T. granulosum* and the present species brings out fairly dissimilarities to consider them identical. While *T. remaki* shows certain features which resembles those of the present form, but judging from the differences of host species and locality, it seems that the both species may be not coincidental too. The specific determination must be leaved to the future study; hence, we refer the present form to *Trypanosoma* sp. provisionally. The trypanosomes of fishes generally not injurious to their host; the present species may be also harmless to eel.

Summary

The trypanosome in the eel collected in eel-culture pond at Yoshida district in Shizuoka Pref. (in the summer of 1956) was studied and described.

The trypanosome differs with the species (*T. granulosum*) infecting in European eel. We refer the species as *Trypanosoma* sp. provisionally.

Literature

DOFLEIN, F. 1928: Lehrbuch der Protozoenkunde, 2 Teil. 5 Auflage. Gustav. Fisher, Jena.

KUDO, R. R. 1921: On some protozoa parasitic in freshwater fishes of New York. J. Parasit., Vol. 7, p. 166.

1923: Skate trypanosome from Woods Hole. Ibid., Vol. 9, p. 179.

1954: Protozoology. 4th Edition, Springfield, Illinois.

LAIRD, M. 1951: Study on the trypanosomes of New Zealand fish. Proc. Zool. Soc., London, Vol. 121, p. 295-308.

LAVERAN*, A. et MESNIL, F. 1901: Sur les flagelle à membrane ondulante des poissons, etc. C. R. Acad. Sc., T. 133, p. 670. *Indirectly cited.

OGAWA, M. 1913: On a trypanosome found in *Triton purrhogaster* (in Japanese). Zool. Mag. (Japan), Vol. 25, p. 518-520.

SCHÄPERCLAUS, W. 1954: Fischkrankheiten. 3 Auflage. Akademie-Verlag. Berlin.

TANAKA, S. 1906: On the taxonomical rank and the characteristics (in Japanease) of trypanosomes. Ibid., Vol. 18, p. 268-272.

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

ON A NEW MYXOSPORIDIAN SPECIES, *THELOHANELLUS CYPRINI*, N. SP., PARASITIC IN THE FIN OF *CYPRINUS CARPIO*

Toshikazu Hoshina and Sadako Hosoda

(Received Jan. 25, 1957)

In the summer of 1956, the senior author had an opportunity to examine young carps, which were presenting small nodules on their caudal fin. Under the microscope, it was proved that the nodules were myxosporidian cysts belonging to the genus *Thelohanellus*. As the present species seems new to science, so we wish here to give the description of it.

We acknowledge to Mr. Tokuo Sano of the Tokyo University of Fisheries for his aid in carrying out the experiment. Further we express our gratitude to Prof. Densaburo Inaba of the same university, for his kindness to place at our disposal the material.

Thelohanellus cyprini n. sp.

(Figs. 1~2)

Habitat.—In the fins especially in the caudal fin of young carps, *Cyprinus carpio*. From the fish-culture pond of the Saitama Prefectural Fisheries Experimental Laboratory (July 7, 1956). Eleven fishes preserved in formol, exhibited the cysts as follows:

No.	Body weight (g)	Body length (cm)	Number of cyst found in....				
			Pectral fins	Dorsal fin	Ventral fins	Anal fin	Caudal fin
1	1.5	3.6	0	0	0	0	5
2	1.4	3.4	0	4	0	0	6
3	1.0	2.9	0	1	0	0	5
4	1.1	3.4	1	0	0	0	2
5	1.9	3.8	2	0	1	1	29
6	1.5	3.4	4	0	4	1	10
7	1.7	3.6	1	0	0	0	16
8	1.0	2.9	4	0	0	0	19
9	1.0	3.0	0	0	0	0	9
10	1.9	3.9	7	2	2	5	12
11	1.1	3.2	14	3	5	5	13
Total			33	10	12	12	126

Vegetative form.—Cysts. They were located in the subepidermal tissue. The cysts are subspherical, varying 0.2 to 1.2 mm by 0.2 to 1.1 mm in size,

somewhat black in surface colour owing to the concentration of host's pigments around them (Fig. 1). They were filled with mature and developing spores. Polysporous.

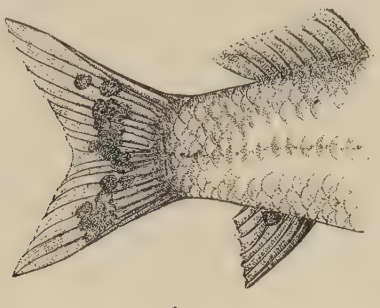


Fig. 1. Showing the cysts in parasitic status.

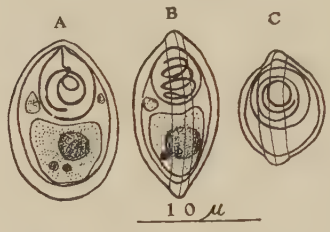


Fig. 2. Showing the spores. Unstained preparation. A, front view; B, side view; c, end view.

Spore.—Viewed from the front, the spores are broadly pyriform, possessing one polar capsule. Lenticular in side and end view. The shell is smooth and moderately thick. The sutual ridge is distinct, but the sutural line indistinct. The polar capsule is ovoid; neck short; coiled polar filament is seen in unstained spores. The sporoplasm contains a comparatively large iodinophil vacuole (3.5～4 μ in dia.) and two compact nuclei (Fig. 2).

Dimensions of spores: length 11～19.5 μ, width 8～13.7 μ, thickness 7.4～11.3 μ, polar capsules 4.7～8.7 μ by 4.7～7.5 μ.

The details including the cysts are compiled in the Table 1.

Table 1. Dimensions of spore and cyst. Notice: Spores in hanging drop preparations were measured by "Schraubenmikrometer."

Measurement Items		Number of spores measured	Range	Mean	Fiducial range (95%)	Standard deviation
Spore	Length	100	11.0～19.5 μ	16.49 μ	16.49±0.252 μ	1.240 μ
	Width	〃	8.0～13.7 〃	10.36 〃	10.36±0.201 〃	0.991 〃
	Thickness	〃	7.4～11.3 〃	9.00 〃	9.00±0.168 〃	0.878 〃
Polar capsule	Length	100	4.7～8.7 μ	7.01 μ	7.01±0.160 μ	0.789 μ
	Width	〃	4.7～7.5 〃	5.99 〃	5.99±0.124 〃	0.610 〃
Cyst	Long axis	100	0.2～1.2 mm	0.53 mm	0.53±0.041 mm	0.201 mm
	Short axis	〃	0.2～1.1 〃	0.47 〃	0.47±0.030 〃	0.196 〃

Remarks

The genus *Thelohanellus* comprises only several members. Of these *Theloh. toyamai* (KUDO, 1920) resembles the present form in the features of spore; inad-

dtion to their host species are same. However there are following differences between them:

Species / Items	*Theloh. toyamai*	Present form
Vegetative form	Cysts. Oval or spherical, 0.067 mm by 0.050 mm in si e Size up 0.2 mm in dia	Cysts. Spherical, 0.2～1.2 mm × 0.2～1.1 mm
Spore	Pyriform, bilateral asymmetry Dimensions: length 15 μ, width 7～8 μ, thickness 5～6 μ Polar capsule pyriform, 7～8 μ by 3～4 μ in size	Broadly pyriform, bilateral symmetry Dimensions: length 11～19.5 μ, width 8～13.7 μ, thickness 7～13 μ Polar capsule ovoid, 4.7～8.7 μ by 4.7～7.5 μ in size
Habitat	Branchiae of carp	Fins of carp

Judging from these differences, they may be not identical. Similary the present form is distinguishable all other related species in every respect. Hence, the present form deserves to be considered as a new species.

Summary

1. A myxosporidian species, *Thelohanellus cyprini* n. sp., parasiting in the fins of young carp, *Cyprinus carpio* is described in the present paper.

2. The infected carps with the myxosporidian species were obtained from the Saitama Prefectural Fisheries Experimental Station, Kazô, Saitama Pref. in July, 1956.

Literature

KUDO, R. R. 1915: On a new myxosporidian from a carp (in Japanese). Zool. Mag. (Japan), vol. 27, p. 516–523.

——— 1920: Studies on Myxosporidia. Ill. Biol. Monogr., vol. 5.

——— 1937: Studies on some protozoan parasites of Illinois. Ibid., vol. 13, no. 1, p. 7–44.

——— 1954: Protozoology. Fourth Ed., Charles C. Thoma. Illinois, U.S.A.

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

HAEMATOLOGICAL STUDIES OF THE CULTURE FISHES IN JAPAN
1. ON THE BLOOD OF EEL

Tokuo Sano

(Received Jan. 28, 1957)

Introduction

Kawamoto (1929–'30) reported following physiological studies of eel. 1. The seasonal variation of the blood constituents of eel. 2. The influence of temperature and of the relative volumes of the red corpuscles and plasma upon the haemoglobin dissociation curve. 3. The microscopic observation of the blood.

So far as I am aware no investigators except perhaps Kawamoto studied on the chemical constituents and the blood picture of Japanese eel. However, the studies on the other chemical constituents except non-protein nitrogen, iron, and haemoglobin are not found in his treatise.

I studied the chemical constituents of the blood of Japanese eel (*Anguilla japanica* T. & S.), viz. sugar, creatinine, urea, calcium, protein, albumin, and globulin. At the same time, I determined the value of haematocrite and the precipitation reaction of blood corpuscles.

Next, concerning the blood picture of eel, there are no studies about the relative number of each kind of leucocytes and mean number of neutrophil leucocyte nucleus. So, I studied according to Kawamoto's classification the percentage of each kind of the leucocytes and the mean number of the neutrophil leucocyte nucleus.

Desiring to determine the various physiological values in healthy condition of all kinds of the pond culture fish in Japan, as the first step of the work, I begun the study from the eel in a season.

I think, such study above-mentioned may be useful for fish-pathology and fish culture.

The study on the other fishes may be proceeded in future.

Previous to going further, my gratitude is due to associate Prof. T. Hoshina, of Tokyo University of Fisheries who granted various helpful advices to me during the course of the study, and also I must acknowledge to Prof. D. Inaba of the same university for his kind supply of necessary materials.

Material and Method

The two groups of the eel cultured at Kawaziri district in Shizuoka Pref. were used for the study, viz. one (Group 1) was kept on starvation for two days before being brought to the laboratory and another group (Group 2) was not starved (passed four–six hours after being given food). They showed no sign of disease, and were measured 49.3–61.3 cm in length, 203.5–360.0 g in weight.

The collection of the blood was carried out quickly from the heart by means of a hypodermic syringe contained a few mg of double-oxalate except the determination of calcium.

The present work was proceeded from July to September, 1956.

The blood sugar was determined by HAGEDORN and JENSEN's method; creatinine by FOLIN and WU's method; urea by HADEN's improved method; serum calcium by SOBEL's method. Albumin and globulin were determined by YOSHIKAWA and SAITO's method.

I used, Hitati serum protein meter to determine serum protein and Westergren's tube for the precipitation reaction of the blood corpuscles. The value of haematocrite was the relative volume of corpuscles and plasma in the Wintrobe's tube revolved for thirty minutes at the rate of 3,000 r.p.m.

The regular smeared preparations of blood stained with MAY-GIEMSA's method were used for the microscopic observations. I observed 500–600 cells in total number to calculate the percentage of neutrophil leucocyte, monocyte, and lymphocyte. The mean number of neutrophil leucocyte nucleus was calculated according to COOKE, PONDER and SUGIYAMA's method.

Result and Disscusion

The results obtained from the group 1 and that of the group 2 are given Tables 1 and 2 respectively.

Table 1. The experimental results in the group 1.

Items \ Results	No. of fish examined	Range	Average	Standard deviation	Fiducial range at 95.0%
Blood sugar	24	66.0~173.6mg/dl	99.43mg/dl	25.914	94.43±11.185
Blood urea	30	2.02~13.80mg/dl	6.19mg/dl	3.341	6.19±1.1654
Blood creatinine	27	1.03~ 1.49mg/dl	1.23mg/dl	0 316	1.23±0.1275
Serum calcium	17	9.36~12.87mg/dl	10.99mg/dl	1.381	10.99±0.732
Serum albumin	10	60.0~76.8%	70.69%	5.252	70.69±3.995
Serum globulin	10	23.0~40.0%	29.31%	5.252	29.31±3.995
Albumin/globulin		1.5~ 3.3	2.52	0.525	2.52±0.400
Serum protein	30	6.6~11.8%	8.00%	1.27	8.00±0.48
Haematocrite value	30	24.0~45.2%	30.4 %	4.57	30.41±1.732
Precipitation reaction of blood corpuscles	14	2.0~2.9 mm/h	2.37mm	0.131	2.37±0.179

Table 2. Showing the chemical constituents in the group 2.

Item \ Results	No. of fish examined	Range (mg/dl)	Average (mg/dl)	Standard deviation	Fiducial range at 95.0%
Blood sugar	7	195.6~286.8	229.66	9.571	229.66±8.625
Blood urea	10	1.10~ 5.47	3.14	1.332	3.14±1.013
Blood creatinine	10	2.91~ 3.23	3.09	0.114	3.09±0.086
Serum calcium	10	11.35~15.25	13.22	1.294	13.22±0.983

Table 3 shows the percentage of each kind of leucocytes and the average of number of neutrophil leucocyte nucleus.

Table 3. The percentage of each kind of leucocytes, each neutrophil leucocyte, and the mean number of neutrophil leucocyte nucleus.

	Percentage of each kind of leucocytes			Percentage of each neutrophil leucocyte			Mean number of neutrophil leucocyte nucles
	Neutrophil (%)	Lymphocyte (%)	Monocyte (%)	N_1* (%)	N_2** (%)	N_3*** (%)	
Range	22.1～66.7	32.9～77.5	0.2～2.5	61.7～98.3	2.5～38.3	4.9	1.02～1.38
Mean	42.76	56.40	0.44	83.25	16.50	0.23	1.168
Standard deviation	13.122	13.761	0.617	10.613	10.524	0.99	0.112
Fiducial range at 95.0%	42.76 ±5.96	56.44 ±6.238	0.44 ±0.280	83.25 ±4.772	16.50 ±4.754	0.23 ±0.44	1.168 ±0.051

* Neutrophil leucocyte of one nucleus. ** Neutrophil leucocyte of two nuclei.
*** Neutrophil leucocyte of three nuclei. 22 preparations were observed.

Field et al (1943) showed that the value of blood sugar is 90–100 mg per 100 cc of blood in the case of healthy carp, so it coincides almost with the value which I obtained from eel (Group 1). The values of healthy man of hunger condition are as follows; blood sugar, 70～110 mg; serum calcium, 9～11.5 mg; blood creatinine, 0.7～1.5 mg, so, those bear a close resemblance to the values of the eel in the same condition (Table 1).

Comparing with chemical constituents in the groups 1 and 2, the great difference are recognized as shown in Table 4.

Table 4. Ratio of the group 1 to the group 2 in chemical constituents.

	Blood sugar	Blood creatinin	Blood urea	Serum calcium
Group 2 / Group 1	2.43	2.51	0.57	1.20

Table 5. Comparison between Onoda's results and result of the present study.

	Neutrohil (%)	Lymphocyte (%)	M.no.n.l.n.*	Observer
Common carp	63.8	34.9	1.05	Onoda ('34)
Crusion carp	70.0	22.0	1.07	〃
Loach	49.0	40.8	1.36	〃
Eel	42.76	56.40	1.168	Sano ('56)

* Mean number of neutrophil leucocyte nucleus.

Concerning the value of the haematocrite, Kawamoto's value: 27.0 at July and 31.0 at August, approximates to my result (Table 1).

Although the precipitation reaction of blood corpuscles should be determined at constant temperature (at 17°C in medical science), owing to insufficient experimental instrument, I was obliged to determine at room temperatures varying 26.5–31.0°C.

On the temperature against the precipitation reaction of the blood corpuscles of fishes, I will study in future.

On the common carp, the crusian carp, and the loach in summer, ONODA ('34) reported that the percentage of the lymphocyte are lower than those of the neutrophil leucocyte, but my result is counter to ONODA's report (Table 5).

Comparing each other with the average of the number of neutrophil leucocyte nucleus in common carp, crusian carp, loach, and eel, it was found as follows: loach > eel > crusian carp > common carp (Table 5).

Neutrophil leucocytes possessing more than four nuclei were not found in eel blood they consisted of one nucleus-cell (83.25%), two nuclei-cell (16.50%), and three nuclei (0.23%) in my observations.

As KAWAMOTO ('30) reported, I could find neither the eosinophil leucocyte nor the basophil leucocyte in eel blood.

Summary

Chemically and morphologically I studied the eel blood in the following two groups. The group 1 was kept on starvation for two days before being brought to the laboratory and the group 2 was not starved (passed four~six hours after being given food).

In the group 1, I determined blood sugar, blood creatinine, blood urea, serum calcium, serum albumin, serum globulin, the value of haematocrite, the precipitation reaction of blood corpuscles, and the mean number of neutrophil leucocyte nucleus (Table 1 and 3).

On the group 2, sugar, creatinine, urea and calcium were determined (Table 2).

Comparing with the results obtained from the groups 1 and 2, it was recognized that there are great differences in the contents of blood sugar, of blood creatinine, and of blood urea between the both groups (Table 4).

There is the following relation between the percentage of each kind of the blood corpuscles. Lymphocyte, $56.44 \pm 6.23\% >$ Neutrophil leucocyte, $42.76 \pm 5.963\% >$ Monocyte, $0.44 \pm 0.28\%$.

As KAWAMOTO reported, also I could find neither the eosinophil leucocyte nor the basophil leucocyte in eel blood.

Literature

FIELD et al., 1943: The blood picture in furunculosis induced by *Bacterium salmonicida* in fish. Archive of Biochemi., Vol. 3, pp. 277–284.

IRIE, T., 1932: On the classification, the speed of movement, and the phagacytose of the leucocyte of crusian carp (in Japanese). Juzenkai-Zassi, Vol. 37, No. 7, pp. 51–56.

KAWAMOTO, N., 1929: Physiological studies on the eel. 1. The seasonal variation of the blood constituents. 2. The influence of temperature and of the relative volumes of the red corpuscles and plasma upon the haemaglobin dissociation curve. Sci. Rep, Tohoku Imp. Univ., 4th Ser. Biology, Vol. 4, No. 4, pp. 635–659.

—— —, 1930: ——————————. 3. Microscopic observation of the blood. Jour., Imp. Inst., Vol. 4, No. 4, pp. 1–6.

ONODA, T., 1934: On the classification of the leucocytes of reptilia and fish (in Japanese). Juzenkai-Zassi, Vol. 40, No. 1, pp. 51–66.

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

PRELIMINARY REPORT ON THE SPAWNING OF GRASS-CARP AND SILVER-CARP IN THE TONE RIVER, JAPAN AND THE DEVELOPMENT OF THEIR EGGS

Densaburo Inaba, Minoru Nomura and Morizumi Nakamura*

(Received Feb. 5, 1957)

Introduction

Since 1947, the three species[1] of Chinese carps, *Ctenopharyngodon idellus*, *Hypophthalmichthys molitrix* and *Aristichthys nobilis*, especially the first two species, have been propagated in the natural waters of the Tone River system including its main flow, Lake Kasumigaura, Lake Kitaura and the Watarase River, etc. (Tange, 1949, Nakamura, 1949, Inaba, 1950 and 1954, Kuronuma, 1954 and Inaba and Nomura, 1956) evidenced by the collection of young fishes of each species. Some phases of the spawning activity of these Chinese carps were reported by Inaba (1956). In summer, 1956 we have again succeeded to secure eggs of grass carp and silver carp in the waters of the Tone River and also in the Edo River which is connected to the former river, and the eggs of two species were reared in out-door fish-pond and glass container in laboratory up to the stages of young fish.

In China, the original habitat of these species, Chen and Lin (1935) and Lin (1935) briefly traced the life history of grass carp; and Lin (1949) reported that the artificial fertilization of grass carp was experimentally made with success in 1936–40 at the Freshwater Fisheries Experiment Station, Kweiping. As to the life history of silver carp, however, our knowledge is so scanty that we have so far been unable even to distinguish morphologically the larval fishes of these species.

The collection of eggs was made in the river water by means of either ordinary plankton net (opening diameter 65 cm) or triangular bag net specially designed (Fig. 1). The triangular portion of the gear is made of silk (or cotton) net, 2.5 or 3.7 m long, which terminates in a silk net cage ($30 \times 20 \times 15$ cm) strengthened by wooden frame. The entire gear is kept afloat by the help of bamboos. The gear was kept in position in the water adjacent to the bank by fastening it to bamboo poles as in Figure 1. During the supposed spawning time the cage was examined at least twice a day, morning and evening.

The eggs or young fish collected were reared in a glass jar or aquarium in laboratory, with frequent change of water. For the feeding of young fish after the disappearance of yolk, our experience showed that the nauplii of the brine shrimp (*Artemia*) and water flea (*Daphnia*) were satisfactory. Three days

* Research Institute for Natural Resources.

1) The recent collection of young black carp (*Mylopharyngodon piceus*) in the lakes Kasumigaura and Kitaura convinces us that the species also naturalized in the Tone River system.

after the hatch-out the supply of green colored water containing abundant plankton organisms (both zoo- and phyto-) helped the growth of both grass carp and silver carp.

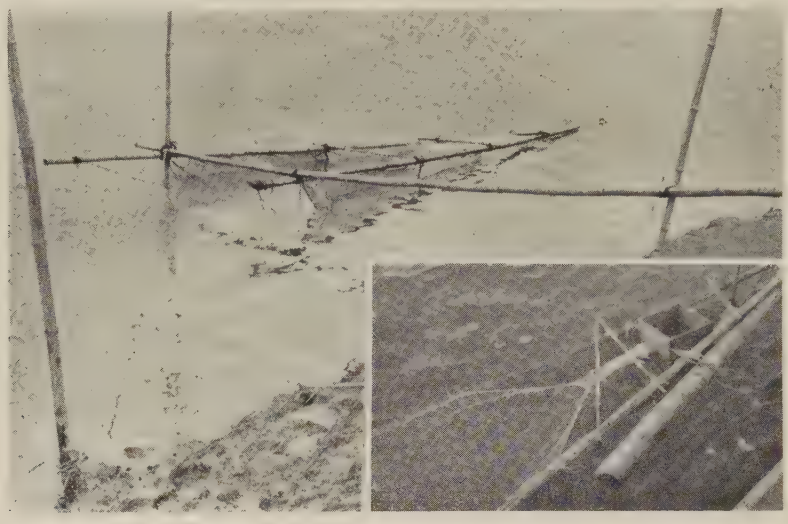


Fig. 1. Collecting bag net set on the bank of the river. Right below, showing the structure of its posterior end.

For the more advanced stage of the young grass carp, the feeding of water flea and mud worm (*Tubifix*) gave better result. The young silver carp was also reared satisfactorily by giving similar food items until they reached the size of 2.5 cm long, but the more developed young of silver carp were found to nibble microscopic algae attached to the inner wall of container or on the aquatic plants placed in the water and also to eat the phytoplankton. It was found that the silver carp in this stage or at a later stage do not show healthy growth if their diet were confined to zoo-planktonic organisms.

The present report is a mere description of our observation since we have not collected enough data for a detailed interpretation of facts. Our colleagues, the fishery scientists, especially of Asiatic nation, are extremely interested in sharing the knowledge and experience which we aquired in our country.

The authors express their hearty thanks to Mr. S. TAKAHASHI, Chief of Commerce and Industry and Fisheries Section of Ibaragi Prefecture, Mr. S. TOMONO, Head of Ibaragi Promotive Fisheries Experiment Station and Mr. S. SUZUKI, Head of Saitama Fisheries Experiment Station, Mr. K. KANZAKI, Head of Mizumoto Branch of Tokyo Fisheries Experiment Station and the members of these Stations for their kind helps given to the authors in the present study. Also we are indebted to Dr. K. KURONUMA, Director, Freshwater Fisheries Research Laboratory, for his helpful suggestions and kind advices.

Status of Spawning

Discovery of actual spawning and spawn. On July 2, 1954, INABA received

news from the Osato Fishermen's Cooperative Association, Kumagaya City, Saitama Prefecture that a large shoal of grass carp were seen congregating in the water of the Tone River. His immediate visit to the spot (Kuzuwata village), made in the morning of following day, failed to witness any sign of grass carp shoal in the water. However, the catch of grass carp by fishermen at this location was evidenced by the fact that scales, mutilated parts of fish body as well as "dried up eggs" remained in a fishing sampan on which he attempted to inspect the fish shoal. INABA's inquiry to local fishermen further revealed that, on the same day (July 2), there was a catch of about 60 big grass carp and also some large silver carp in the neighbouring waters. These fishes, female dominating in number, measured 10 to 15 kg in weight and about 1 m long. This is the first evidence of spawning of Chinese carp in Japanese waters witnessed by a fishery scientist in Japan. The exact spot and time of their spawning were clarified on this occassion.

Fig. 2. The Tone River system showing the spawning site of Chinese carps and the localities where eggs and fry were collected. Spawning site, enlarged in Fig. 3, is encircled by square and the spots of collection are indicated by arrows. Names of towns and villages given are frequently referred to in the text.

In early part of July (4 to 8), in the same year, INABA and NOMURA attempted a painstaking effort to obtain eggs or fry of Chinese carp in river waters in the main flow of the river at Toyoshima village, Ibaragi Prefecture, about 120 km downstream from Kuzuwata community where actual spawning of the carp was witnessed. On the evening of July 8, they succeeded to collect 14 eggs and 7 larval fish at this spot, which were believed to be those of grass carp which were just hatched out. The identification of these specimens was entirely based on the report by CHEN and LIN (1935) and LIN (1935) but left a certain amount of doubt due to the fact that our specimens were not studied alive but

examined in the state of preservation in formalin. Another collection of fish fry was successfully made at the same spot, 1 to 4, August, 1954 and these fishes were proved to be the young of grass carp by rearing them in laboratory.

The local fishermen stated that in the year 1955, the spawning of Chinese carp took place in the same region of the river and in almost the same time of the year. Our study, however, was not made extensively due to unavoidable circumstances, and our activity was confined in the collection of live eggs in the river water. The eggs, some 230 in number, were secured in the river water at Otone community near Kazo City, Saitama Prefecture. From the varied developmental stages demonstrated by these eggs we presumed that the spawning water where eggs are discharged is not so far apart from the spot where we collected eggs. The spawning site thus presumed falls on the same region where witnessed the spawning in 1954.

In 1956, from June 2 to July 8, we were given better opportunities than in the previous years for the study of spawning of these carps. A group of fishery workers consisting of INABA, NOMURA and staff of Saitama Prefecture Fisheries Experiment Station finally succeeded to witness Chinese carps (both grass and silver) spawning in action. The real spawning of these species which we actually saw took place in the main flow of the Tone River extending from Kuzuwata down to Kawamata, but their activity was believed to have been centered 1 to 2 km measuring from the intake of each Minuma-dai and Kasai irrigation canals (Fig. 3).

On the other hand in this year (1956) we were placed fortunately in the successful collection of eggs which proved to be those of grass carp and silver carp by tracing their development. These eggs were collected by the staff of Mizumoto Branch of Tokyo Fisheries Experiment Station, amateur biologist Mr. S. UMEZAWA and the authors in the waters at Toride Town on the Tone River as well as at Nagareyama and Matsudo along the Edo River which is a tributary of the Tone River (Fig. 2). The eggs, totalling more than 11,000 in number, were obtained on 8 and 9 July, and the study of their development described in the following pages of the present report by NAKAMURA was made based on these eggs.

Spawning behavior. Our observation of actual spawning of carp is by no means satisfactory mainly due to the fact that the activity was witnessed only in 1956 and keen observation was not made possible because of the single spawning pair in abroad area of water surface.

The spawning action of the grass carp may be described as follows: a female, usually followed by one to three males, swim around near the surface of water rolling and rubbing their body, often jumping out of water in the course. We are of opinion that such action of carp is a spawning behavior of the species, however, we were unable to observe the moment of discharge of eggs by female nor the ejaculation of sperm fluid by male in their action as such.

Such congregation of male and female fish was observed sporadically and simultaneously in the river water with no restriction referring to the location and the flow or depth of water at least during the peak of their sexual activity. The spawning of grass carp in a day lasted from early morning (5 a.m.) to early evening (7 p.m.), but their higher activity was observed at dawn and twilight.

The temperature of river water was measured 20.9° to 21.9°C during the act of their spawning in 1956, but the range of temperature was not restricted to as such. The highest reading of our thermometer recorded 30.4°C at 3 p.m. on June 10 in the year of 1955, when the Tone River saw an unusual drought.

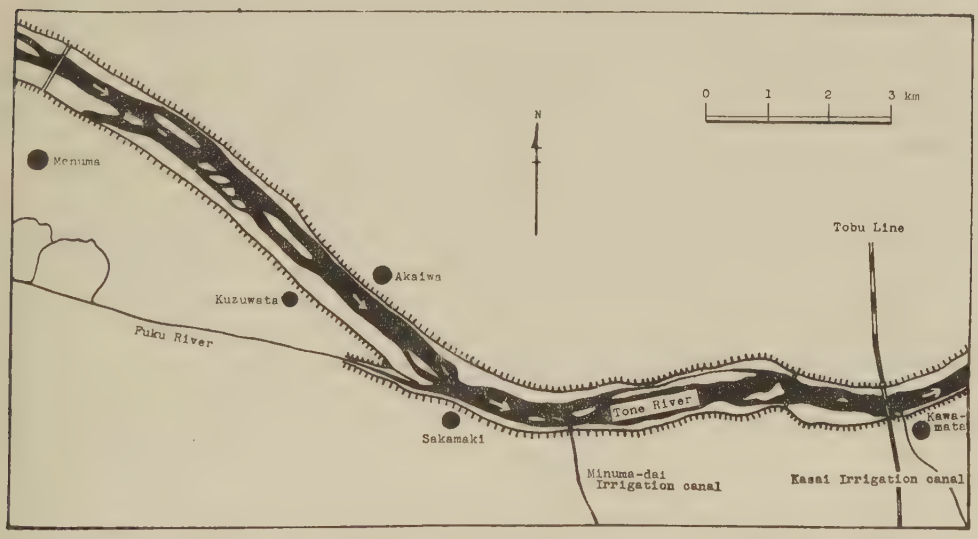


Fig. 3. Spawning site of grass carp and silver carp on the main flow of the Tone River, the activity was witnessed in the water extending from Kuzuwata down to Kawamata. See text.

Topography and Hydrography. The River Tone, the third longest river in Japan flows from west to south-east on Kanto Plain and opens into the Pacific ocean at Cape Inubo. The communities of Sakamaki and others, where the spawning takes place, are located about 160 km up from the Cape Inubo and about 40 km NNE of Tokyo City. The width of the river near Sakamaki is some 800 m from dyke to dyke and the river bed extend into meadowy flat of 100 m or more which lies immediate below the dyke on both sides. The true river bed holding the water flow is measured 200 to 400 m in width (Fig. 3). The depth of water in flow is variable by season, but in the ordinary flow in summer months, the deepest spot measures about 2.5 m. The variability of the water level in this region of the Tone River is well shown by the survey of Kawamata Field Station of Ministry of Construction and the daily changes of the water level in June and July in the three years are shown in Figure 4.

The bottom of the river in this region consists mostly of coarse or fine sand and in some places there are coarse gravel or pebble with sand. The aquatic vegetation is very scarce except some bushes of water-oat and other plants scattered along the shore.

The annual change of water temperature is between about 4° to 29°C and the monthly average is reported by the survey of Central Meteorological Observatory (Table 1).

Table 1. The monthly average of water temperature (°C) in the Tone River, at Fusa near Toride. Source: C.M.O. 1953, p. 18.

Year Month	1949 V	〃 VI	〃 VII	〃 VIII	〃 IX	〃 X	〃 XI	〃 XII	1950 I	〃 II	〃 III	〃 IV	Average
Water Temp.	19.5	23.2	27.7	27.6	21.0	19.4	9.5	6.9	5.0	6.2	11.2	13.6	15.91

The chemical analysis of water in the Tone River was extensively made by KOBAYASHI (1955) at various spots and frequency, the results extracted in Table 2. The locality Kawamata in the table is close to the spawning site of the carp and the chemical components there will represent that in the spawning water; the other locality Toride locates far below Kawamata in the river (Fig. 2).

The pH value of the water, probably showing the most reliable indication for the understanding of water, was measured 6.5 to 7.1 with average of 6.9 (Table 2). Our measurement of the value, however, ranged 7.1 to 7.2 in either water of Sakamaki and Otone, July 1956. Reviewing the chemical analysis of the water (Table 2) we may not find any significant character considering general features of our rivers and streams in this country. The single exception is, however, found in the content of SO_3 which shows 26.1 mg/l in average at Kawamata, and the concentration is much higher than at Toride (16.8 mg/l) and Sawara (15.9 mg/l). Whether the concentration of SO_3 has any significance to the spawning of grass carp is hardly predictable at the present moment. The concentration of other chemical components will undoubtly be lowered at the time of heavy rain (cf. data in May 1953, Table 2) which has significant bearing on the spawning of carp as noted elsewhere in the present report.

Table 2. The chemical properties (mg/l) of the waters of the Tone River (After Kobayashi, 1955).

Locality	Date	CaO	MgO	Na_2O	K_2O	SO_3	Cl	SiO_2	P_2O_5	Total solids	Suspended solids	pH	Hardness
Kawamata	May 15, '53	9.6	3.1	4.5	1.11	12.6	3.9	15.1	0	62	61.9	6.5	1.4
	July 15, '53	21.0	4.0	8.0	1.74	23.2	6.8	26.6	0	104	19.2	7.1	2.7
	Jan. 15, '54	22.7	4.3	11.2	2.44	35.5	13.7	42.5	0.02	138	16.8	6.7	2.9
	mean (14)*	19.7	4.9	8.6	1.77	26.1	8.8	27.8	0.01	109.3	26.8	6.9	2.8
Toride	mean (8)*	18.6	5.5	7.5	1.76	16.8	5.3	23.0	0.01	97.9	18.5	7.1	2.5
Sawara	mean (13)*	18.2	4.9	10.2	1.86	15.9	7.7	21.1	0.01	95.4	18.7	7.1	2.5

* Number shows the frequency of measurement; Hardness 1=CaO 1 mg/100 cc.

As to the level of river water and grass carp spawning in Chinese West River CHEN and LIN (1935) and LIN (1935) stated that the spawning of the fish requires the following conditions; raising of water level over 4 feet which takes place less than 12 hours prior to the activity, the velocity of water flow records 12,000 to 21,000 feet per hour (or 1 m/s to 1.8 m/s), and the spawning site in the river frequents usually at the junction of two flows and its immediate upper reach finds faster flow.

According to our experience on the Tone River it was usual that the spawning of either grass carp or silver carp takes place when the water level was raised 2 to 4 feet over normal level of the days with rain fall one day or two before (Fig. 4). The transparency of water measured by Secchi's disc measured, therefore, as low as 10 to 25 cm in these occasions.

Exception to such a general state the spawning of carp witnessed by us on June 2, 1956 was not followed by immediate fluctuation of water level as shown in Figure 4. In this occasion the water with normal level showed transparency of 90 cm and temperature of 18.2°C, also, the collection of eggs in the water made simultaneously proved the two species, both grass carp and silver carp, participated in the spawning. In fact our colleagues of Saitama Prefecture

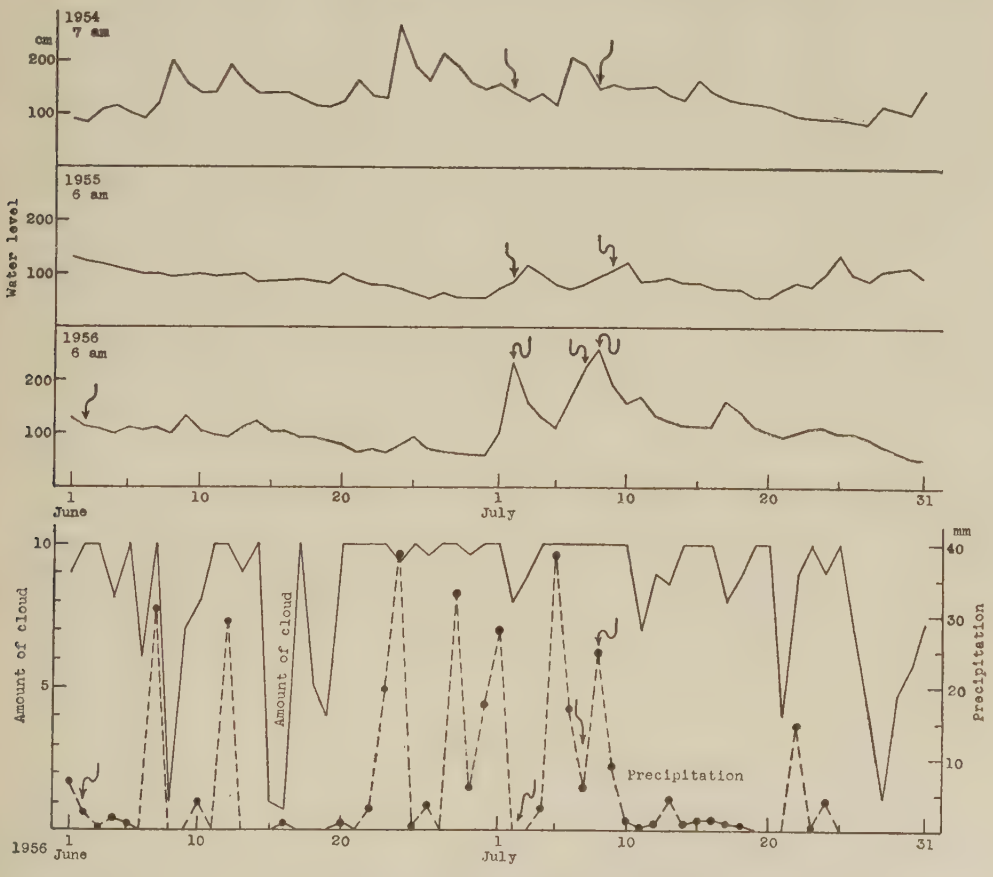


Fig. 4. Fluctuation of water level measured at Kawamata in 1954, 1955 and 1956 by Ministry of Construction Field Station (upper three figures); the reading of levels was read on the water guage placed in water, thus, not indicating the actual depth of river water. The bottom figure shows the precipitation and amount of cloud surveyed by Kumagaya Meteorological Observatory in 1956. The arrows show the date of spawning of grass carp and silver carp witnessed by either actual observation or collection of eggs in water. See text.

Fisheries Experiment Station succeeded, for the first time in Japan, the egg stripping and artificial fertilization of silver carp.

The approximate flow of water, measured along the shore of spawning site on July 2, 1956, recorded 0.6 to 1.2 m/s on the surface and 1.03 to 1.05 m/s in the subsurface water 50 cm deep.

Ovarian eggs. LIN (1935) reported that a female grass carp weighing 7 kg carry 100,000 eggs in China. One of our example, collected at Fujioka town along the Watarase River on July 21, 1954, measured 880 mm in total length and 7,135 g in weight, carrying ovary and eggs as shown below:

	Left	Right	Total
Weight of gonad (g)	163	134	297
Length of gonad (mm)	295	280	—
Number of eggs per gram	1,646.1	1,617.7	—
Estimated number of eggs (thousands)	268	217	485

The ovarian eggs in this specimen measured about 1 mm in diameter, the largest with the dimension of 1.5 mm; the size frequency of the eggs in ovaries was nearly 50—50 with the mode at 1 mm. This fact leads us to believe that the grass carp discharge her eggs repeatedly with some intervals but not in a single spawning act, though we now lack the evidence to predict the frequency of discharge. CHEN and LIN (1935) also hold similar interpretation on this subject.

On the ovarian eggs of silver carp we have no our own data, but will quote the measurement made by the Saitama Prefecture Fisheries Experiment Station (June, 1956): a female 815 mm long and weighing 12 kg carried ovarian eggs estimated at 800 thousands, which were stripped nearly completely.

Development of eggs and fry

The materials of eggs we secured originated either by stripping from female fish or collection made in river waters, and the tracing of the development of the eggs to fry was also made based on the samples collected in natural waters and those reared in laboratory. The developmental stages of fish were observed and traced under the categories: egg, embryo in egg, hatching-out, prolarva, postlarva, and juvenile, in order.

Grass carp

Egg. The matured eggs were obtained from a female fish (920 mm long and 8.25 kg in weight, July, 1956), which measured 1.7 to 1.9 mm in diameter under preservation (5% formalin), there eggs swelled up to about 4 mm in diameter some 60 minutes after the immersion into fresh water, but no embryonic development occurred. The dimension of live eggs in river water varied 3.8 to 5.5 mm in diameter as far as our materials concerned[1]. The nearly spherical egg is non-adhesive and demersal in nature; color in life may be described pale

1) LIN (1935) reports that fully matured eggs in ovary measure 2.0 to 2.5 mm in diameter, but they swell up to the size of 5–6 mm 2 or 3 hours after fertilization.

blue with slight tinge of yellow, but the yellowish tinge grow clearer after the egg swelled up in water[1]).

Embryonic development. The earliest development of embryo we could observe was the D-shaped stage (Fig. 5, A and Pl. I, A). The embryo, 2.2 to 2.5 mm long, is characterized by elongated optic vesicle and myotomes counted some 5. The eggs with embryos in such stage were placed in a glass container with water of 19.5° to 22°C in temperature and their development was traced (Fig. 5, A to E) up to the stage of hatching-out which took place between 26 to 29 hours later.

Two hours later of the D-shaped stage the myotomes increase to about 25 and the yolk grows longer (Fig. 5, B). The embryo grow to 3.8 mm long, 3 hours later, with myotomes counting about 28 and the yolk resembling a pear in shape (Fig. 5, C); prior to and in this stage the embryo move rhythmically, with head and tail up-and-down at the hinge placed in the middle of back, such movement of body parts lasting 2 to 3 hours.

One to two hours later the yolk changes into a dumb-bell shape (Fig. 5, D); the rhythemical movement of body, similar in pattern but bend ventrally and much vigorous than in the previous stage, counts 70 to 80 a minutes, and the bending of body is made so acute to make often the head and tail nearly touch with each other. The movement of body in such a mode lasts about 2 hours, then, the moving of body is confined to the tail which now shows horizontal jerking and lasts another two hours. Toward the end of this stage the moving of embryo becomes so vigorous that it often turns up-side-down inside the perivitellin cavity.

Within one or two hours after that, the tail grows longer, the yolk resembles tadpole and the myotomes increase to about 37, the embryo now measuring 4.8 mm long (Fig. 5, E); in this stage both tail and head show active moving. From this stage of embryonic development to the time of hatching-out, requiring 15 to 16 hours, the embryo continue the growth and its moving action is further

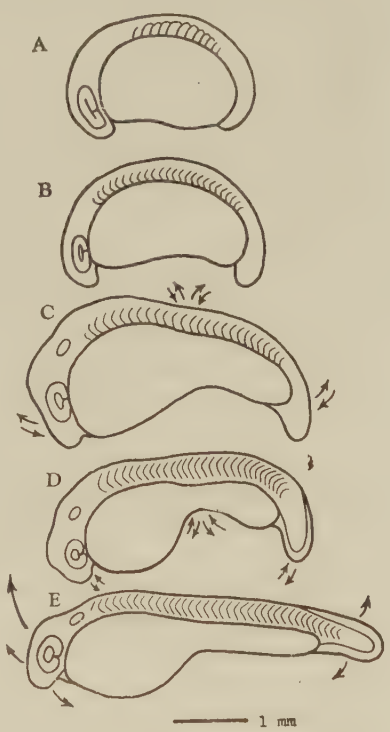


Fig. 5. Semidiagramatical drawings of development of grass carp embryo, showing the relative growth of bodily parts and the pattern of moving shown by tail and head (arrows indicating the direction). A, the earliest stage of development found in our collection of eggs, believed 13 to 15 hours after fertilization (according to Lin and Chen (1935)); B, stage 2 hours after A; C, stage 3 hours after B; D, stage 1–2 hours after C; E, stage 5–6 hours after D.

1) Verbal expression of the life color of egg is extremely difficult, and such as those given here are far from the true coloration which our sight can catch.

magnified, and the hatching-out of egg is usually preceded by the swimming action of embryo within the perivitellin cavity. An embryo measured 5.1 mm in length just before the hatching-out (Pl. I, B).

Prolarva. The description of the developmental stage was made based on 3 specimens of varied total length.

1. The smallest specimen, 5.1 mm, (Pl. II, B) was obtained on July 4, 1956; the egg collected in the Edo River. July 3, and reared in container up to the hatching-out (at 21° to 23°C) and killing for observation. The slender body is characterized by elongated head and trunk and short tail, the vent opening at the point 2/9 part of body from the rear end. The yolk is chicken egg shaped, tapering posteriorly. The head, ventrally inclined, inserts slightly below the mid-line drawn on the yolk. The eye in greater part is colorless and transparent; a small amount of melanophores are shown near lower margin of symphysis of optic cup. The myotomes (ca. 28+13=41) are not well differentiated on tail part.

The larval grass carp in this stage tend to lie with their sides on the bottom of container, but often enter swimming motion shaking its tail and head laterally; sometimes, they swim up to the surface of water in head-up position and immediately return to the bottom. Apparently they did not show the behavior to hang on the wall of container or on floating matter in the water, as often demonstrated by the larvae of common carp, gold fish and some of other cyprinid fish.

2. A prolarval of 6.4 mm (6.7 mm in live) long was obtained on June 5, killed 24 hours after hatching-out (Pl. II, C). The long head and trunk are still characteristic feature of the fish, also the tail is short. The pectoral fin shows its original structure. The myotomes count about 30+11=44.

Still sedentary in behavior they tend to lie on the bottom of container as in the previous stage, but with a larger interval, they swim around in the water.

3. The largest prolarva 7.4 mm long was obtained on July 8, 1956, killed 3 days after hatching-out. In the grass carp of this stage, the head is further differentiated feature by the development of opercles and mouth parts; the lower jaw, still incomplete, and its anterior tip reaches only below the anterior edge of eye. The iris is gold and pupil is black. The pectoral fin is yet membranous in structure.

The head and back of the fish now reflect greenish yellow tinge; the melanophores, starlike in shape lying just below the skin, develope especially on the back of head, suborbital region, the back of trunk, base of pectoral fin, surrounding heart, anterior part of yolk, marginal region between the dorsal part of yolk and the lateral myotomes, ventral edge of post-anal myotomes, and on tail part of future hypural bone. The myotomes, counted about 30+14=44, are still incomplete and the 3 pieces in the rearmost are not well discernible.

The fish at this stage still tend to lie on the bottom, and sporadically leaves the bottom for short swimming.

Postlarva. The description of this developmental stage was made based on four specimens of varied length.

1. A fish 8.1 mm in total length was secured on June 11, 1956, killed 7

days after hatch-out. The yolk is now completely absorbed, and the air-bladder is recognized. The lower jaw is slightly inferior to the upper. The distribution of melanophores is seen throughout the head, trunk (except ventral margin) and tail part which grow future fin rays; the marginal region between digestive tract and lateral myotomes is lesser pigmented than in other bodily parts noted above. The myotomes count about 28+12=40.

The grass carp in this stage take normal position in water and swim around slowly near the bottom in container; they start feeding on brine shrimp, small water flea and the like given by our hand.

2. The second specimen of this stage (9.1 mm in total length) was obtained on July 21, 1956, killed 16 days after hatch-out (Pl. III, B). The two jaws are now equally developed. The notochord turns up in the posterior end, accompanied by the growth of original constraction of hypural bone and about 8+7=15 caudal fin rays. The dorsal fin fold emerges at the position of future dorsal fin with the development of the original fin rays numbering 5. The anal fin also stared its formation on 4 pieces of future rays. The iridocyte layer begins to appear in such parts; laterally to heart and on body cavity laterally to and facing the digestive tract. The myotomes count 28+13=41.

The young grass carp in this stage swim slowly in the bottom layer of container seeking the food. The swimming action of the fish, made in linear fashion, resembles that of common carp and wild gold-fish.

3. The third specimen of postlarval stage (12.1 mm long) was obtained on July 23, killed 18 days after hatching-out (Pl. III, C). The caudal fin now with two lobes recognized assumes a normal shape and bears 10+9=19 fin rays, a given formula to species. The median fin fold also differentiates into dorsal and anal fin, the former with 8 rudimentary rays and the latter 7 well recognized. The pelvic fin also shows its original construction. The air-bladder is divided into antero- and postero-chamber.

The largest specimen of postlarval stage (18.6 mm long) was obtained on June 24, 1956, killed 20 days after hatching-out (Pl. III, D). The fish of this stage or size is identified as grass carp without much difficulty. The head and back of the trunk are brownish yellow with greenish tinge, and the ground color fades out toward the belly. The iridocyte layers are formed on opercle, pre-opercle, branchiostegales and peritoneum.

The median fin fold still remains on the ventral edge of body extending from below the posterior edge of pectoral fin to the vent as well as on the dorsal and ventral edge of caudal peduncle. The dorsal fin acquires the given fin formula of IV, 8, but the anal, having II, 8 rays, has the anterior one or two unbranched ray undeveloped. In the pectoral fin which is still fan shaped, the 8 upper rays are well formed and rest of the rays remaining undifferentiated. The pelvic fin is still short but bears normal fin formula of I, 6. The myotomes count 29+12=41.

The grass carp of this size are good swimmer in the mid-layer and bottom in the active persuit of food organisms.

Juvenile. The grass carp of juvenile stage were described based on three specimens of varied length.

1. The smallest fish (21.3mm long) was obtained on August 4, 1956, killed one month after hatching-out (Pl. III, E). The fish of this size is well characterized by the scales which cover the entire body with the exception of ventral edge of trunk; the scales on lateral line count about 38. Fins are all completed, each with given fin ray counts. A remnant of fin fold is seen, only in a short space, posterior to the pelvic insertion. The iridocyte layer are found on post-orbital, opercle, the subsurface of mid-lateral part of trunk and on the peritoneum.

The grass carp of this size are good swimmer and actively search food organisms in mid-layer or deeper water; their motion of swimming suggests the beginning of their migration in the natural water. Such natatory behavior of grass carp of this size will be compared to that of silver carp in a clear contrast, because, the silver carp juvenile, preferring to stay in surface water, swim round under the incessant motion.

2. The specimen 36.7 mm long was obtained on August 30, killed about 50 days after hatching-out (Pl. I, D). The scales are now complete, fin fold is not recognized and the various parts of body are completely made, if not its proportional length of juvenile character.

The grass carp of this size begin to feed on aquatic plants, such as Duck-weed and Ditch moss in the container or aquarium in addtion to the plankton organisms in the water.

3. The largest juvenile (67.3 mm long) was obtained on August 30, 1956, killed about 55 days after hatching-out (Pl. I, E and E′); this fish was reared in an out-door pond, with 10 m^2 surface area. Except its size the fish completely attain the external characters of grass carp, and they now feed on silk-worm-pupae-meal, rice-bran and the like given by our hand.

Silver carp

Egg[1] *and embryonic development.* For the tracing of those stages we have not been given chances either in field or in laboratory, and the following accounts were quoted from a mimeograph circulated by the Saitama Prefecture Fisheries Experiment Station. On June 2, 1956, a female fished at Sakamaki measured 81.5 cm long and weighed 10.95 kg, carrying fully matured eggs 1.6 to 1.9 mm in diameter (preserved in formalin). The eggs of this female fish were fertilized normally under artificial condition, and they swelled up about 2.5 times in diameter one hour after fertilization, and hatched out within 41 to 48 hours after in the water 18° to 22°C in temperature.

Prolarva. The description of this stage was made based on two specimens of varied length and origin.

1. The fish, 5.3mm in total length, hatched out at 6p.m. on June 4, 1956, the egg fertilized artificially on June 2, at Sakamaki (Fig. 6. A). The slender body carries oval shape yolk which tapers backward. The melanophores develope very poorly on the outer edge and just in front of symphysis of optic cup within. The myotomes, counting about 24+14=38, are not well differentiated toward the tail.

1) The eggs of silver carp were studied in detail by the workers of Saitama prefecture Fisheries Experiment Station, and will be reported upon in near future.

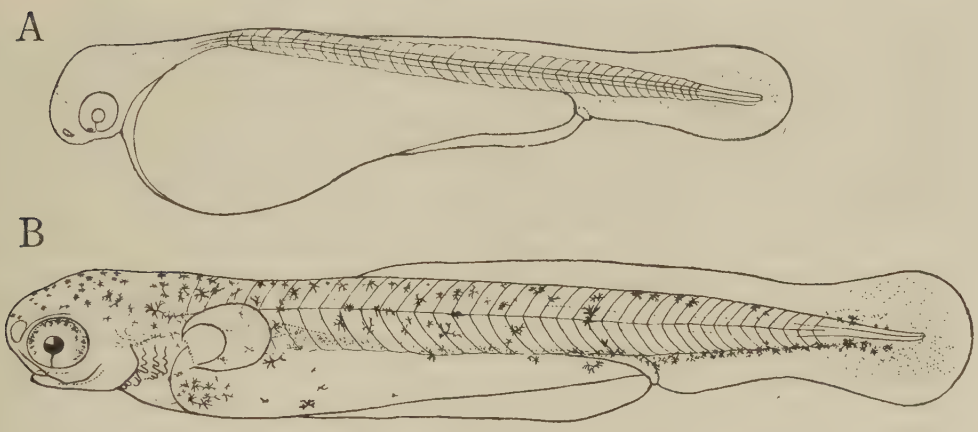


Fig. 6. Prolarval stage of silver carp.
A. 5.3 mm in total length, immediate after hatch-out.
B. 7.3 mm in total length, three days after hatch-out.

The young of silver carp at this stage may be distinguished from grass carp in such characters as noted below under microscope:

	Grass carp	Silver carp
Pre-anal myotomes	28–30	24
Head	less differentiated	more differentiated
Yolk	slenderer	robust
Head and trunk compared to tail	larger	much larger
Fin fold	narrower	broader

2. The fish, 7.3 mm in total length, was obtained on June 7, 1956, killed 3 days after hatching-out; the egg was collected in river water at Matsudo and reared in laboratory (Fig. 6. B). The head now well differentiates with opercles developed and two jaws working; the lower jaw is shorter than the upper. The body is slender and carries pectoral fin fan-shaped. The iris is golden and pupil black. The melanophores are seen developed on the back of head and body, side, ventral margin of post-anal myotomes, vicinity of heart and on the side of yolk; they are also seen scattered on the back of yolk facing lateral myotomes. The myotomes count 24+14=38. The silver carp in this size is also distinguishable from grass carp by the number of pre-anal myotomes.

The fish tend to lie on the bottom and sometimes move forward by shaking its tail.

Postlarva. The description of this developmental stage was made based on 4 specimens, of varied length, reared in laboratory or in fish pond.

1. The fish, 8.8 mm in total length, was obtained on June 11, 1956, killed 7 days after hatch-out (Pl. IV, A). The yolk is completely absorbed. The air-bladder is now seen. The mouth opens anteriorly on head, with two jaws of equal length. The melanophores, grown smaller than in previous stage, are

found evenly on head and trunk, some spreading on to specified portions of fin fold where median fins are expected to grow and on pre-anal portion. The development of melanophores on fin fold, lasting to the later stage of juvenile, is highly specific and unique to silver carp among all the fish species referred to the Family Cyprinidae dwelling our freshwaters[1]. The xanthophores also grow abundant on the back of head and trunk, resulting the greenish yellow color reflected on live fish. The myotomes count 24+14=38.

The fish attain the normal position in the water and swim around in pursuit of food such as brine-shrimp given by our hand.

2. The fish, 9.0 mm in total length, was obtained on July 21, 1956, killed 10 days after hatch-out (Pl. IV, B). The notochord turns up on the end, carrying about 9+8=17 caudal rays. The dorsal fin shows its sign of development on fin fold, and the ventral fin fold, anterior to vent, grow very broad. The pelvic fin does not yet show the origin. The melanophores, now covering the entire surface of body save the belly, result dark ground color on whole body of fish. The pigmentation on fin fold, believed specific to this species, become further clean on this stage and is reconized on pre-anal (posterior 1/3) part, dorsal and anal fin, caudal fin membranes and on peduncle. The myotomes count 23+14=37.

The young silver carp of this stage swim around incesantly in surface or mid-layer of water seeking planktonic algae such as *Microcystis*. Our experience found that they devoured brine shrimp and grew well, but those fed mainly by water flea (*Daphnia pulex*) were nearly starved to death.

The grass carp and silver carp in this size, if they are observed in the same body of water, may be identified by the swiming motion and the coloration. The silver carp swim around incesantly (or inch-by-inch motion) in the surface water, and they look darker due to the concentrated melanophores on caudal fin and ventral fin fold.

3. The fish, 12.3 mm in total length, was obtained on July 23, 1956, killed 12 days after hatch-out (Pl. IV, C). The depth of body grows higher than in the previous stage. The air-bladder divided into two parts. The caudal fin, carrying given formula of 10+19, is completed and forked as in grown-up fish. The dorsal fin, separated from fin fold, carries 9 rays; the anal fin, well developed but continued posteriorly to fin fold, counts 11 rays. The pectoral fin is still round in shape, with no differentiated rays. The pelvic fin shows its original structure on the belly and strait below the insertion of dorsal fin. The ventral fin fold is still very broad. The myotomes count 24+14=28. The occurrence of melanophores is nearly same as in the previous stage, but the dense pigmentation on vertical fins, especially those on the margin of caudal as well as those on ventral fin fold characterize the silver carp of this size. The iridocyte layer, developed on opercle and body cavity, result the silvery reflection on whole body of fish; the melanophores on those bodily part subside underneath the iridocyte layer.

The behavior of silver carp in this size resembles to that in the previous

1) We were not given chance to trace the development of *Aristichthys nobilis* which might possess such pigmentation as noted.

stage; the fish, forming a school of 2 or 3 individuals, swim around in the surface water of pond and seek the planktonic algae, which they devour. The young silver carp at this size is further differentiated from grass carp in their coloration and in general behavioristic character.

4. The fish, 19.0 mm in total length was obtained on July 7, 1956, killed 34 days after hatch-out (Pl. IV, D). The general body configuration is nearly completd to characterize the species; the body is further deepened; each fin is nearly completed; iridocyte layer is better developed. The caudal fin is deeply forked, bearing well developed rays. The dorsal fin, growing higher, carries the given formula of III, 7 rays; the anal also bears formulated number of rays (III, 11). The pectoral fin, not yet fully grown, bears only 8 upper rays differentiated. The pelvic fin bears formulated number of rays (I, 7). The fin fold all disappears from the body except that on the belly, which grows narrower than in the previous stage. The ground color on head and back is yellowish brown with greenish tinge, and the side and belly are brilliantly reflected by gold due to the development of iridocyte layer, which are found on the lateral side of head and other parts of trunk. Each vertical fin is darkened toward free margin. The melanophores on ventral fin fold spread anteriorly beyond pelvic fin.

The behavior of silver carp in this size is similar to that shown in the previous stage, but its moving is observed further active.

Juvenile. The description of this developmental stage was made based on 3 specimens of varied length.

1. The fish, 23.7 mm in total length, was obtained in a fish pond on August 11,1956, killed 31 days after hatch-out (Pl. IV, E). The general shape of the body, not much differentiated from the previous stage, resembles more closely the adult fish. The ventral fin fold, especially anterior to the pectoral insertion, become narrower, but its basal part grows thicker changing into a "keel" structure. The pectral fin is now fully grown and bears the given number of 13 rays.

The young silver carp up to this size showed excellent appetite on the brine shrimp given by our hand, but high mortality was noticed on these fish. The fishes reared in pond showed active swimming in dense shoal often bending their body, in the surface water; often a number of fish demonstrated simultaneously a behavior resembling the "gulping air", though no sign shortage of oxygen surveyed in the pond water. The many specimens obtained in the pond were found to have swallowed planktonic algae in full content of digestive tract.

2. The fish, 35.8 mm in total length was obtained in fish pond on August 30, 1956, killed some 50 days after hatch-out (Pl. I, G). The scales are now seen developed on the surface of body over the body cavity and side of caudal peduncle. The iridocyte layer, covering whole body surface except head and back, effect further golden reflection on the body. The general habit of silver carp in this size does not vary much from that observed in the previous stage.

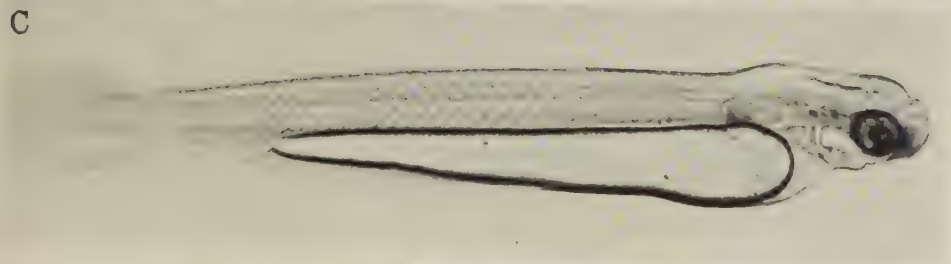
3. The fish, 51.3 mm in total length (Pl. I, H), was obtained under the same condition as the previous specimen. The scales are completely formed covering the nearly whole surface of body. The general shape of the fish assumes that of the adult, except the position of eye which still lies on the horizontal body axis and the proportional length of various bodily parts.

Literature

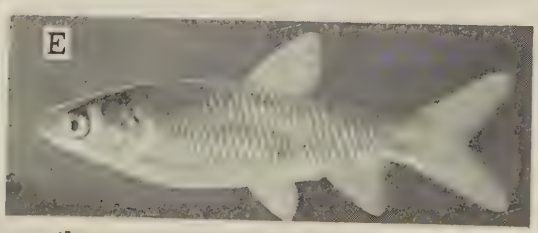
1. CHEN, C. B. and LIN, S. Y. 1935: Fish fry industry of China (in Chinese). Rep. Chekiang prov. Fish. Exp. Sta. 1 (4).
2. INABA, D. 1954: Imported species and its propagation in fresh-water pisciculture—The propagation of imported fishes from China at the River Tone and Ara (in Japanese). Rakusui, 3. 1954.
3. ——— 1956: On the propagation of grass carp in the River Tone. 28th Meeting Lakes, River and Pisciculture Cong. at Kanagawa.
4. ——— and M. NOMURA 1956: On the digestive system and feeding habits of young Chinese carps collected in the River Tone, Jour. Tokyo Univ. Fish., 42 (1).
5. KOBAYASHI, J. 1955: Chemical investigation of the water of Rivers in Japan (report 3). The nature of water in the Kanto districts (in Japanese). Nogaku Kenkyu 43 (1).
6. KURONUMA, K. 1954: Do Chinese carps spawn in Japanese water? Proc. IPFC 5th Meeting. Section II.
7. ——— 1955: What I hear about the spawning of grass carp in the Tone-River, Japan. (mimeograph). IPFC 6th Meeting.
8. LIN, S. Y. 1935: Life history of Waan Wu, *Ctenopharyngodon idellus* (C. et V.). Lingn. Sci. Jour. 14 (1) and 14 (2).
9. ——— 1949: Notes on fish fry industry of China. IPFC Proc. 1st Meeting.
10. NAKAMURA, M. 1949: Notes on the Chinese cyprinids, *Ctenopharyngodon idellus* (Cuv. et Val.) and *Hypophthalmichthys molitrix* (Cuv. et Val.) propagating in Japan (in Japanese with English summary), Misc. Notes Res. Inst. Nat. Res. No. 14.
11. Oceanogr. Sect., C. M. O. 1953: On the chemical properties of several river waters on Kwanto Plain, Japan (in Japanese with English abstracts) Kaiyo Hokoku 3 (1–2).
12. Saitama Pref. Fish. Exp. Sta. 1956: On the propagation of silver carp and grass carp in Saitama Prefecture (mimeograph, in Japanese). Saitama Pref. Fish. Exp. Sta.
13. TANGE, M. 1949: On the propagation of *C. idellus* and *H. molitrix* in the waters around Lake Kasumi and Kita (in Japanese). Res. Material No. 12, Res. Soc. Fish. Agency.
14. YOSHIKOSHI, Y. 1939: Report on the fish fry industry of West River. Canton Bureau of Agriculture and Forestry. (in Chinese translated in Japanese). Jour. of Fish. No. 43.

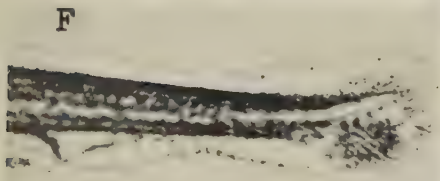
A

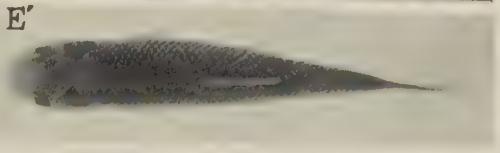
B


C

D


E


F


E'

G

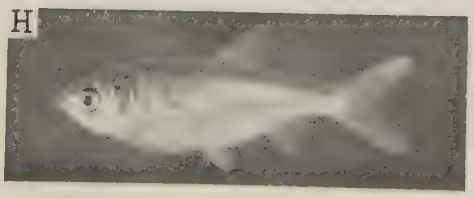

H

PLATE I.

A. An egg of grass carp, 5.0 mm in diameter, collected in river water at Toride, July 3, 1956.

B. The same, but collected at Matsudo on July 4, 1956.

C. Prolarval stage of grass carp, one day after hatching-out reared in a glass container, July 1956. ×18

D. Juvenile of grass carp reared in fish pond, measuring 36.7 mm in total length.

E. The same, measuring 67.3 mm.

E'. The same, seen dorsally.

F. Tail of postlarval silver carp of 8.5 mm long; note thick pigmentation carried by the species comparing to grass carp (C. Pl. III. B. and Pl. IV. B.).

G. Juvenile of silver carp reared in fish pond, measuring 35.8 mm in total length.

H. The same, measuring 51.3 mm.

PLATE II.

Egg and young of grass carp in prolarval stage, collected in river water at Toride and Matsudo. The development of the young was traced in glass container.

A. Egg measuring 5.0 mm in diameter; the same material was taken by photograph (Pl. I, A).

B. Prolarval stage, 5.1 mm in total length; immediate after hatch-out.

C. The same, 6.4 mm; a day after hatch-out.

D. The seme, 7.4 mm; 3 days after hatch-out.

PLATE III.

Developmental stages of grass carp traced by rearing in laboratory and fish pond.

A. Postlarvel stage, 8.1 mm in total length; 7 days after hatch-out.

B. The same, 9.1 mm; 16 days after.

C. The same, 12.9 mm; 18 days after.

D. The same, 18.6 mm; 20 days after.

E. Juvenile stage, 21.3 mm in total length; one month after hatch-out.

PLATE IV.

Developmental stages of silver carp traced by rearing in laboratory and fish pond. The original material is the same as those traced in Plate II.

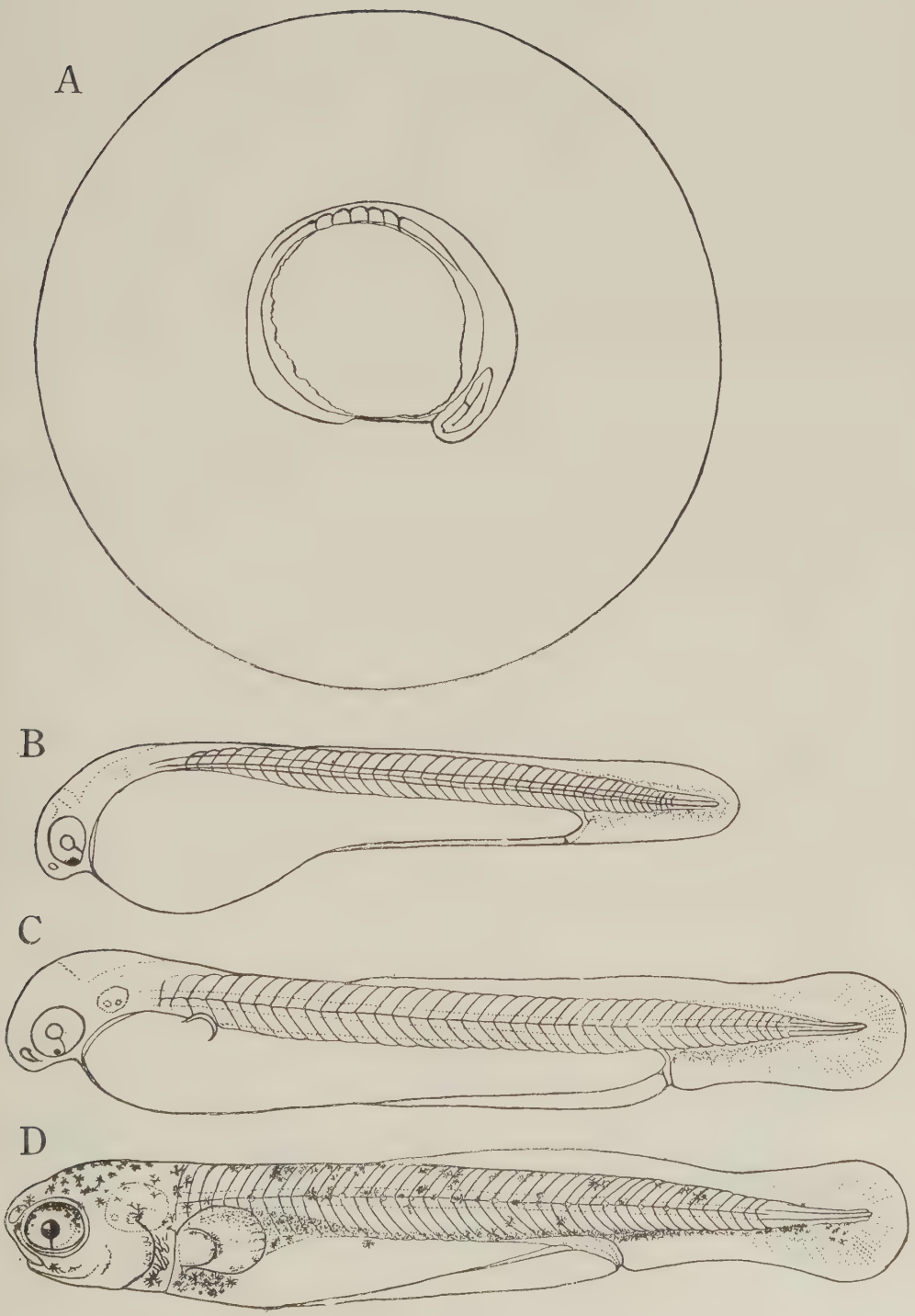
A. Postlarval stage, 8.8 mm in total length; one week after hatch-out.

B. The same, 9.0 mm; 10 days after.

C. The same, 12.3 mm; 12 days after.

D. The same, 19.0 mm; 34 days after hatch-out, the specimen reared in an aquarium in door.

E. Juvenile stage, 23.7 mm in total length, 31 days after hatch-out, the specimen reared in a fish pond.


A
B
C
D

A
B
C
D
E

A
B
C
D
E

Journal of the Tokyo University of Fisheries, Vol. 43, No. 1, 1957

DESCRIPTIONS OF SOME NEW GOBIOID FISHES OF JAPAN, WITH A PROPOSITION ON THE SENSORY LINE SYSTEM AS A TAXONOMIC CHARACTER

Kazunori TAKAGI

(Received February 4, 1957)

The gobioid fishes here described were found on South Japan and collected by Mr. Yosie DÔTU of Kyushu University, who sent them to me for identification. They are represented by six species found to be new to science of one new genus and six new species: **Amblyeleotris japonicus*, sp. nov., *Ctenogobius dotui*, sp. nov., *Callogobius shunkan*, sp. nov., **Stigmatogobius minutus*, sp. nov., *Paleatogobius uchidai*, gen. et sp. nov., and *Chaenogobius scrobiculatus*, sp. nov. The asterisks mean that the genera concerned have not been introduced from Japan.

In this study, I try to apply on the goby classification a form of the sensory line system subject to the anterior and posterior lateral nerves (CORDIER 1954: 228), to say more precisely, the disposition of the sensory canal pores, out opening of the sensory canals of the system. Those canals occur on head, and are to be controlled by the former nerves in the said fish.

Since SANZO (1911), directed by an analogous principle in RAFFAELE (1889)'s luminous organ study on the Myctophid fishes, recognized the systematic value of the disposition of the system in the gobioid fishes, some essays have been achieved by taxonomists on the application of the disposition of chiefly the pit-lines, another form of the system. The canal pores appear to have hardly drawn attention of them, except of a few workers, though SANZO (1911: 325) refers already to the disposition of both canals and "*forami mucosi*," or canal pores, as well as that of the pit-lines, which must, said he, shed light on true relationships of the gobioid fishes. By the way, the close relationship between the system in question and the luminous organs has been discussed. (GÜNTHER 1887, Y. LE DANOIS 1956, e.g.)

I must herewith offer my deep gratitudes to Prof. Itsuo KUBO, under whose supervision the present study was made. His hearty thanks due to Prof. Keitaro UCHIDA at Kyushu University, who recommended me this study, and to Mr. Yosie DÔTU, who kindly put his valuable specimens at my disposal. I feel grateful sincerely also to Prof. Kiyomatsu MATSUBARA at Kyoto University, who encouraged me during the study, and to those gentlemen who help me to consult literature or in various ways: Prof. Marinus BOESEMAN at Rijksmuseum van Natuurlijke Historie, Leiden, Prof. Enrico TORTONESE at Museo Civico di Storia Naturale "Giacomo Doria," Genoa, Prof. FEIDER at Academiei Republicii Populare Romîne, Jassy, Ass. Prof. Kentaro YANO and Mr. Nobuo EGAMI of Tokyo University, Dr. Bertil LEKANDER of Statens Skogsforskningsinstitut, Stockholm, and

Messrs. Ryo KATASHIMA and Akihiko INABA of Hiroshima University, and Fujio YASUDA of Tokyo University.

The specimens treated here with are taken during 1943–54 from Kagoshima, Saga or Fukuoka Prefectures, Kyushu, as mentioned below, and deposited by Mr. DÔTU in the Laboratory of Fishery Biology of the university, except for a smaller syntype of *Callogobius shunkan*, sp. nov., which is in the Kyushu University. Also seen some other specimens of their related species.

Considering that the goby classification seems to rest systematically, say in chaos at least in part even in these days, and to require first more possibly precise study or description for each species, as says already FAGE (1914) or BORCEA (1933), I show here the diagnoses in detail, in the sense, of glossohyal bone, pelvic fin, anal papilla, and canal pores of the sensory line system, as well as other descriptions. Of these taxonomic characters, the glossohyal bone is found by me (1950) to have an important systematic value in the gobioid fishes. The pelvic fin also seems to claim much more interest of us. On the sensory line system will be given below some lines. For the examination of canal pores the binocular microscope with special lamp, made by Carl Zeiss, Jena, is to be of good service to us.

The sensory line system in the fishes takes many terms by workers[1]. I follow here on the appellation of this system STENSIÖ (1947) and LEKANDER (1949), who shed some new lights upon the matter. The terminology of each canal pore is already proposed by SANZO, but I dare present here new names seen below, after the model of the Myctophid study of FRASER-BRUNNER (1949). They are probably suitable for the recent scientific results.

The systematic value of the disposition of the system is presumably to be laid much more stress on by goby workers, while after SANZO (1911) some of them coincide in with him and insist its applicability. (FAGE 1914, 1915a, b, MORTARA 1918, LAMBERTENGHI 1919, DE BUEN 1924, 1930, ILJIN 1927, 1930, BORCEA 1933, and HARRY 1948.) As for the other fishes than the goby, besides, the system is treated with on the classification of the Cyprinodontid, Percid, Callionymid, Tetraodontid, Liparid, Hexagrammid, and Batrachoidid fishes, for instance. (GOSLINE 1949, HUBBS and CANNON 1935, OCHIAI *et al.* 1955, Y. LE DANOIS 1956, MATSUBARA and IWAI 1954, etc.)

The sensory line system has probably interested in the goby study, principally as a way to elucidate the complicated relationships among the European *Gobius* fishes and related (FAGE 1915: 20). It is apparently acceptable for its applicability to be extended beyond the *Gobius* group, because HARRY (1948) obtains a sufficient result by applying it to the classification of the *Bunaka* fishes and related, Eleotridae.

The sensory line system in the fishes is generally known to consist of

1) Sensory line system: Lateral, or Latero-, sensory system, Lateral sensory line system, Lateral line system (Système, or Réseau, de la ligne latérale), Acoustico-lateral system, Système, or Organes, muco-sensoriel(s), Neuromast system, Lateral line sense organs, Cutaneous sense organs (Hautsinnesorgane), Kopfseitenorgane; (Sensory) canal pores: Open pores, Pores muqueux; Pit-lines: Pit organ lines, Papilles cutanées, Sensitory papils, Papillae, Organes cyathiformes (Organi ciatiformi), génipores.

three series of the canals, grooves, and pit-lines. On the system study of the gobioid fishes, the taxonomists seem to have given too much importance only to the interspecific variations of the pit-line disposition, though proving some varieties of it in each species. The canal pores also represent, as mentioned below some, remarkable and more stable specific differences in their disposition, and they must be important for the wide study, as well as, or in the first step, rather than the pit-lines.

The disposition of the system, especially of the pit-lines, are sometimes beyond easy discernment on living or preserved specimens, because of thick mucous layer frequently covering them on the body. For the system study Sanzo (1911) shows exactly a method for clearing and relevation of the canal pore and pit-line disposition, which may be effective, as insures already Fage (1914). The method, as suggested by de Buen (1930: 123), is not always applicable in taxonomic study yet, because principally it makes specimens injured by chemical substance. As far as my trial goes, the canal pores are found to be recognized more easily before chemical treatment.

On the other hand, the sensory canals are known to precede embryologically and palaeontologically to the pit-lines (Stensiö 1947: 101, Lekander 1949: 122), if it "begins to develop and becomes fully developed rather late, ..." (Lekander op. cit.) Another proposition of Lekander (1949: 110–111) may be worth setting forth here. After observation of his examined species, he says "it would appear...that they [the canals] form in a specific order and that many characteristics, with regard to their formation, recur from one species to another," and he continues, "in this respect, the Cyprinids constitute a uniform group, while *Nemachilus* essentially differs from the 'Cyprinid type.'" On the Cyprinodontid genera, besides, Gosline (1949) proves three "major groups" in the canal structure.

As for the gobioid fishes proper, Taranetz (1943) refers to the canal pores in his *Gymnogobius* study, but his description of them for the genus is covered by the family character suggested formerly by Sanzo (1911) and others. It is interesting, however, he (p. 400) recognized the intergeneric difference of its disposition between this genus and *Chasmichthys*, and points out that "the structure of pores and mucous canals on head probably differs from that of the genus *Gymnogobius*," after examination of malpreserved specimens of the latter genus. As an intergeneric difference between *Chaenogobius* and *Chasmichthys*, Tomiyama (1936: 39) refers to "a pair of pores on posterior part of interorbital space" (Itop, below, Fig. 1). de Buen (1930: 133) refers exactly to its disposition in a synonymic discussion, and comes to a conclusion that *Pomatoschistus* (*Bubyr*) *caucasicus* (Kawrajsky) differs from *P.* (*Ninnia*) *canestrini* (Ninni), by presence or not of some canal pores (Itoa and Sot, below). (See Table 2.) He considers apparently the canal pore disposition as specific character. Koumans (1931: 44) also suggests the importance of the canal structure, but he has not published the study on it in detail.

As noticed by Borcea (1933: 8), it should be cautioned to overate the weight of the said character, but, considering he refers apparently to pit-line disposition only, it may be permitted to expect to some extent a progress to the

present goby systematics by the study on future of the system in question.

These are the reason why in this study the canal pores are at first examined, instead of the pit-lines. Nevertheless, the canal structure seems inevitable on, but remains to further scrutiny.

The canal pores of the gobioid fishes are known, since SANZO (1911), to hold as a rule twelve positions on head. One in nasal region[1], two on interorbital space, three each behind eye, on preopercular margin, and on upper opercular margin, but sometimes more than twelve, when they takes probably a part of the pit-lines, or less, when some of them are more or less closed or absent.

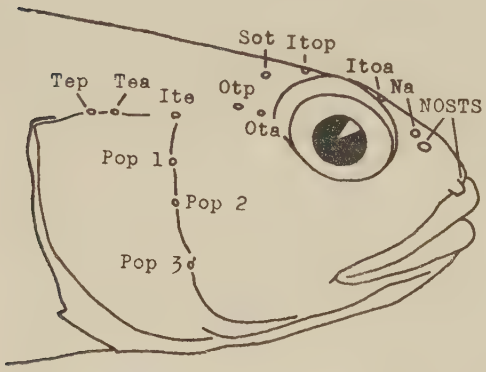


Fig. 1. Typical disposition of the sensory canal pores in the gobioid fishes (*Gobius* Type). See text for abbreviations, except NOSTS, nostrils.

The pores on these positions will be sketched as follows (Fig. 1). The canals on which the pores concerned open are in parentheses.

Na, the nasal pore (supraorbital canal), in a pair[1], namely, one each on the sides; its position moves by species, from above the interspace of nostrils to behind the posterior nostril. It is scarecely seen to be closed when some others are present. (SANZO's definition: σ).

Itoa, the anterior interorbital pore (supraorbital canal), single, that is, medialy unique, or else in a pair, and occurs in both cases with single Itop, below; its position appears invariable on anterior half of the interorbital space. (SANZO's: λ)

Itop, the posterior interorbital pore (supraorbital canal), single or in a pair; the single Itop occurs usually[2] with single or paired Itoa, and the paired Itop occurs always without Itoa: its position moves by species to-and-fro on posterior half on the said space. (SANZO's: k)

Sot, the supraotic pore (supraorbital canal), in a pair; its position is behind the upper hind margin of eye, and above the oculoscapular groove, if present. (SANZO's: ω)

Ota, the anterior otic pore (infraorbital canal), in a pair; its position moves by species along the above groove, from below to just behind the eye. (SANZO's: α)

Otp, the posterior otic pore (infraorbital canal), in a pair; its position moves by species on anterior half of the groove. (SANZO's: β)

1) In our unidentical specimens of a gobiid species, stomach contents of a conger, *Astroconger myriaster* (BREVOORT), from the Tokyo Bay, I prove the nasal pores occurring in two pairs. I suppose more than one, at least two, paired pores are recognizable in the nasal region of some gobioid fishes, especially of the Indo-Pacific species. The definite positions of the canal pores are to be of thirteen, in the case.

2) See reference to ILJIN's two genera, below.

Ite, the intertemporal pore (probably temporal part of infraorbital canal), in a pair; its position moves again on posterior half of the said groove to before the pore Tea, below. (SANZO's: ρ)

Pop_{1-3}, the preopercular pores (preopercular canal), in a pair each, arranged from above transversely along the preopercular margin. Pop_3 sometimes keeps away below from the others. One of them, if present, may be found to disappear by species; it is Pop_1 or Pop_2, but it happens very hardly. (SANZO's: γ, δ, and ε)

Tea and Tep, the anterior and posterior temporal pores (temporal canal), in a pair each, occur each other always dependently; their positions not variable on upper opercular margin. (SANZO's: ρ', and ρ'')

Above all, some accessory or abnormal pores have been occasionally seen in some species, near Sot or Tep, as figured by SANZO (1911), and BORCEA (1933). It may be said rare occurrence.

The canal pore disposition observed in 53 species of Europe and Japan may be summarized as shown in Table 1. Of the European species, the figures of SANZO (1911), FAGE (1914, 1915b), DE BUEN (1930) and BORCEA (1933) are available to know the disposition. According to DE BUEN (1930: 133), two other genera, *Bubyr*, and *Knipowitschia*,[1] erected by ILJIN (1927, 1930) based on *Gobius caucasicus* KAWRAJSKY and *Gobius longicaudata* KESSLER respectively, seem to be very unique by their canal pore disposition. Relied on the descriptions by DE BUEN, the pores of them may be marked as seen on the Table 2, where their disposition are the same as seen in all European species, except that of the pores referred to on description. The single Itop, therefore, occurs without Itoa in this case, if my interpretation is exact. The paired Itop does not appear to be mentioned by ILJIN.

Table 2. Probable canal pore disposition in *Pomatoschistus* (*Bubyr*) *caucasicus* (KAWRAJSKY) and *P.* (*Knipowitschia*) *longicaudata* (KESSLER). See remarks on Table 1.

Canal pore	Na	Itoa	Itop	Sot	Ota	Otp	Ite	Pop_1	Pop_2	Pop_3	Tea	Tep
P. (*B.*) *caucasicus*	1	—	(1)	1	1	1	1	1	1	1	1	1
P. (*K.*) *longicaudatus*	—	—	(1)	1	1	—	1	1	—	1	—	—

From the results on the Table 1, I recognize in the disposition the following seven types, based chiefly upon the occurrence of the pores Na, Itoa, and Itop (Table 3). It may be suitable to mention here that the supraorbital canal, on which the above three pores occur, as well as infraorbital and mandibulo-preopercular canals, drows chief attention of LEKANDER (1949: 111) on the canal formation in the fishes. It seems to show that these canals on the anterior part of head are more characteristically important than the posterior temporal

1) While DE BUEN (1930) treats these genera as subgenera of *Pomatoschistus*, as mentioned above, the taxonomic status of them seems to rest in further examination, viewed from the present angle.

Table 1. Canal pore disposition of some Japanese gobioid species and available foreign species. Species name based mostly by DE BUEN ('30); 1), subgenera of *Gobius*, acc. KOUMANS ('31); 2), =*Coryphopterus*, acc. KOUMANS ('31); 3), = *Gobius affinis* KOLOMB., see DE BUEN ('23); 4), =*Gobius capito* CUV. et VAL., see DE BUEN ('30). 1, in a pair; (1), single; —, closed; ?, probably 1 in Na, (1) in both Itoa and Itop, —in Sot. These ? come from authors' rough sketches. 5), No Sot but deep groove here in my examined specimen. Fg, source of this disposition table, figures of BORCEA (Ba), DE BUEN (Bn), FAGE (F), HARRY (H), and SANZO (S). Gps, groups of european species by FAGE (F), and SANZO (S).

Species	Canal pore disposition												Fg	Gps	
	Na	Itoa	Itop	Sot	Ota	Otp	Ite	Pop_1	Pop_2	Pop_3	Tea	Tep		S	F
Gobiidae:															
Type I.															
1. *Lesueuria*[1] *lesueuri* (RISSO)						(many pores)							S	AI	I
2. *L. friesi macrolepis* KOLOMBATOVIC						(many pores)							S	AI	I
3. *L. sanzoi* (DE BUEN)						(many pores)							Bn		
Type II.															
4. *De'tenosteus*[1] *quadrimaculatus* (CUV. et VAL.)	1	(4)		—	1	—	1	—	—	—	1	1	S	AII–1	II–1
Type III.						(+many pores)									
5. *Rhinogobius similis*(nec GILL) JOR. et SNY.	1	1	(1)	1	1	1	1	1	1	1	1	1			
6. *Stigmatogobius minutus*, sp. nov.	1	1	(1)	1	1	—	1	1	1	1	—	—			
Type IVA.															
7. *Pomatoschistus*[1][2] (*Iljinia*) *microps* KRÖYER	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		III
8. *Proterorhinus*[1] *marmoratus* (PALLAS)	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		
9. *Gobius* (*Zebrus*) *zebrus* (RISSO)	1	(1)	(1)	1	1	1	1	1	1	1	1	1	S	BIII	IV
10. *G.* (*Gobins*) *niger* LINNÉ	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		IV
11. *G.* (*G.*) *niger jozo* LINNÉ	1	(1)	(1)	1	1	1	1	1	1	1	1	1	S	BIII	IV
12. *G.* (*G.*) *auratus* RISSO	1	(1)	(1)	1	1	1	1	1	1	1	1	1	S	BIII	IV
13. *G.* (*G.*) *vittatus* VINCIGUERRA	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Bn		IV
14. *G.* (*Macrogobius*) *geniporus* CUV. et VAL.	1	(1)	(1)	1	1	1	1	1	1	1	1	1	S	BIII	IV
15. *G.* (*M.*) *cruentatus* LINNÉ	1	(1)	(1)	1	1	1	1	1	1	1	1	1+2/1	S	BIII	IV
16. *G.* (*Zostericola*) *ophiocephalus* PALLAS	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		IV
17. *G.* (*Babka*) *gymnotrachelus* KESSLER	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		
18. *G.* (*Messogobius*) *batracocephalus* PALLAS	1	(1)	(1)	1	1	1	1	1	1	1	1	1+1	Ba		
19. *G.* (*Apollonia*) *melanostomus* PALLAS	1	(1)	(1)	1	1	1	1	1	1	1	1	1+1	Ba		
20. *G.* (*Ponticola*) *platyrostris* (nec PAL.) NORDM.	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		
21. *G.* (*P.*) *cephalarges* PALLAS	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		
22. *G. bucchichi* STEINDACHNER	1	(1)	(1)	1	1	1	1	1	1	1	1	1	S	BIII	IV
23. *G. constructor* NORDMANN	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		
24. *G. lacteus* NORDMANN	1	(1)	(1)	1	1	1	1	1	1	1	1	1	Ba		
25. *Ctenogobius dotui*, sp. nov.	1	(1)	(1)	1	1	1	1	1	1	1	1	1			

26. *Acentrogobius campbelli* (JORDAN et SYDER)	1	(1)	(1)	1	1	1	1	1	1	1	1	1			
27. *Callogobius shunkan*, sp. nov.	1	(1)	(1)	1	1	1	1	1	1	1	1	1			
Type IVB.															
28. *Buenia reticulatus*[3] (CUVIER et VALENCIENNE)	1	(1)	(1)	1	1	—	1	1	1	1	1	1	S	AII-1	II-2
29. *Coryphopterus*[1] *fravescens* (FABRICIUS)	?	?	?	1	1	—	1	1	1	1	1	1	Bn		
30. *Pomatoschistus ruuthensparri* EUPHRASEN	1	(1)	(1)	1	1	—	1	1	1	1	1	1	S	AII-2	III
31. *P.* (*Englauligobius*) *kneri* STEINDACHNER	?	?	?	1	1	—	1	1	1	1	1	1	F		III
32. *P.* (*E.*) *quagga* HECKEL	1	(1)	(1)	1	1	—	1	1	1	1	1	1	S	AII-2	III
33. *P.* (*Iljinia*) *pictus* A. W. MALM	1	(1)	(1)	1	1	—	1	1	1	1	1	1	F		III
34. *Gobius* (*Macrogobius*) *paganellus* LINNÉ	1	(1)	(1)	1	—	1	1	1	1	1	1	1	S	BIII	IV
35. *G.* (*M.*) *cobitis*[4] PALLAS	1	(1)	(1)	1	—	1	1	1	1	1	1	1	S	BIII	IV
36. *G. depressus* KOLOMBATOVIC	1	(1)	(1)	1	1	1	1	1	1	1	—	—	S	BIII	IV
37. *Pomatoschistus* (*Pomatoschitus*) *minutus* (PAL.)	1	(1)	(1)	—	1	1	1	—	1	1	1	1	F		III
38. *P.* (*P.*) *minutus lozanoi* DE BUEN	?	?	?	—	1	—	1	1	1	1	1	1	Bn		
39. *Gobius elongatus* CANESTRINI	?	?	?	?	1	—	1	1	1	1	1	1	F		
40. *G. ferrugineus* KOLOMBATOVIC	1	(1)	(1)	—	1	—	1	1	1	1	1	1	S	AII-2	
Type V.															
41. *Paleatogobius uchidai*, gen. et sp. nov.	1	—	1	—	—	1	—	1	1	1	—	—			
42. *Chaenogobius isaza* TANAKA	1	—	1	—	1	1	—	—	—	—	—	—			
43. *C. urotaenia* (HILGENDORF)	1	—	1		—	1	1	—	—	—	—	—			
44. *C. scrobiculatus*, sp. nov.	1	—	1	1	—	—	—	—	—	—	—	—			
45. *C. castanea* (O'SHAUGHNESSY)	1	—	1	—	—	1	—	—	—	—	—	—			
46. *C. heptacantus* (HILGENDORF)	1	—	1	—	—	1	—	—	—	—	—	—			
47. *C. murorana* (JORDAN et SNYDER)	1	—	1	—	—	1	—	—	—	—	—	—			
Type VI.															
48. *Pomatoschistus* (*Ninnia*) *canestrini* NINNI	—	(1)	(1)	—	1	—	1	1	—	1	—	—	S	AII-2	III
Sicydiaphiidae:															
Type VII.															
49. *Aphya minuta* RISSO	—	—	—	—	—	—	—	—	—		—	—	Ba		
Eleotridae:															
Type III.															
50. *Ophiocara porocephala* (CUVIER et VALENCIENNE)	1	1	(1)	1	—	1	1	1	1	1	1	1	H		
Type IVA.															
51. *Amblyeleotris japonicus*, sp. nov.	1	(1)	(1)	1	1	1	1	1	1	1	1	1			
Type VII.															
52. *Mogurnda obscura* (TEMMINCK et SCHLEGEL)	—	—	—	??1[6]	—	—	—	—	—	—	—	—	H		
53. *Bunaka gyrinoides* (BLEEKER)	—	—	—	—	—	—	—	—	—	—	—	—	H		
Benthophilidae:															
Type VII.															
54. *Benthophilus stellatus* SAUVAGE	—	—	—	—	—	—	—	—	—	—	—	—	Ba		

canal. In the gobioid fishes no canal pore has been observed on the mandibular canal.

Type I (*Lesueuria* Type): Many pores occur on head; pores on the tweleve positions not recognized independently.

Type II (*Deltenosteus* Type): Many pores present; all definite ones not recognized independently, except of severals. Intermediate form between Types I, and III–VII.

Generally speaking, in the following Types III–VI the pores are not seen but on the definite twelve positions, and the nasal pores occurring in a pair (Na) accepted, if present.

Type III (*Rhinogobius* Type): Na, paired Itoa and single Itop present; some other pores seen or not by species.

Type IVA (*Gobius* Type): Na, Itoa and Itop, both single, present; the other pores also fully developed.

Type IVB (*Buenia* Type): Na, Itoa and Itop, both single, present; the other pores not fully developed, that is, some of them more or less absent on the definite positions. This is a relatively indefinite type, for including the species, on which are absent one or two paired pores on positions behind eye.

Type V (*Chaenogobius* Type): Na, no Itoa and paired Itop present, and some other pores may be more or less absent.

Type VI (*Ninnia* Type): no Na, and Itoa and Itop, both single, present; some other pores absent.

Type VII (*Aphia* Type): no pores seen on body.

Moreover, if DE BUEN (1930)'s references from ILJIN is complete, as mentioned above, the subgenera *Bubyr* and *Knipowitschia* are to be included each other into another type respectively.

Table 3. Canal pore disposition types. See remarks on Table 1.

Types	Na	Itoa	Itop	Remarks
I				many pores
II	1	(4)		+many pores
III	1	1	(1)	+9 pores or less
IVA	1	(1)	(1)	+9 pores
IVB	1	(1)	(1)	+less pores than 9
V	1	—	1	〃
V′	1	—	(1)	probable occurrence
VI	—	(1)	(1)	+less pores than 9
VI′	—	—	(1)	probable occurrence
VII	—	—	—	no pore

From Table 1 we may draw consequences as follows:

1) The occurrence of these types does not correspond to the present phylogenetic supposition (STARKS 1930: 72–73, MATSUBARA 1955: 807–808); types are seen in families.

2) It is apparently shown yet that the canal pore disposition is effectively used to some extent as taxonomic character.

3) The said disposition may be considered as somewhat stable generic character, because of occurrences of *Gobius* species in Types IVA, B, and of *Chaenogobius* species homogeneously in Type V.

4) Intergeneric difference is, then, probably proved between *Ctenogobius* and *Rhinogobius*.

5) Notwithstanding, European genera or subgenera related to *Gobius*, especially *Pomatoschistus*, are more or less heterogeneous in this point of view. The genus *Gobius* proper is to be naturally put in Type IVA, because of *G. niger* in this type of disposition.

The phylogenetic considerations of these types all remains in this study. As for the relationship between the types in canal pore disposition and shape of glossohyal bone, the results to this extent do not show but that in types of more pores occurring are seen degenerated types of the bone.

Sanzo (1911) and Fage (1915) classify the European species into some types, based upon the pit-line disposition only, and on the other general characteristics as well as the said feature, respectively. In these works, Sanzo refers to 17 cited species in the Table 1, and Fage to mostly overrupped 22 ones. The types concerned, I–IVB, and VI, grouped up here, appear to be more accordant with Fage's groups, than Sanzo's.

Descriptive

1. *Amblyeleotris japonicus*, sp. nov.

New Japanese names: Date-haze

Dorsal fin VI–I–13; anal fin I–13, pectoral fin 18. Scales 82 in a lateral low, except of last one on caudal base, 32 in a transverse row.

Body elongate, compressed, height 6.2 in length, 8.5 in total length. Head obtuse, as high as breadth, 3.8 in length, 5.2 in total length. Eye 4.8 in head, interorbital 0.5 eyediameter. Nostrils without tube, though anterior one opening frontwards protrudes with membraneous lid; anterior one well behind upper lip. Snout obtuse, short, 0.7 eyediameter, tip below (or before in lesser two specimens) inferior margin of pupil. Mouth horizontal. Lower jaw a little prominent. Maxillary extends at most to below posterior margin of pupil. Teeth pointed, curved, in 3–4 rows, outer row enlarged; on each side in both jaws 2–3 canines, although at least one of them possibly small, 3/1–1/2–1/3. Tongue rounded or a little pointed at tip, furnished rather densely with tubercles on dorsal surface (at least in major specimens), attached at posterior half to mouth floor. Glossohyal bone of narrow fan-shape, truncated on distal margin. Gill-opening wide, proceeds ventrally, isthmus very narrow, branchiostegeal membranes hardly separated by narrow space. Gill-rakers short, compressed, furnished with many minimal spines; 9 of developed ones on lower arch, and others reduced as tubercles, which are exclusive on upper arch. Behined symphasis, a pair of pocket, in which a pit-organ is probably furnished with, opens backwards. Anal papilla

well distinguished in shape between both sexes, of small tubercle in male, well developed pap-like in female. Caudual peduncle 1.9 in head, height 1.4 in its length.

Canal pores fully developed, all without tube opening. Na opens on inside of middle of both nostrils. Itoa single, as well as Itop; these well separated. Sot present. Ota and Otp present, the latter opens rather backwards, in posterior half between Ota and Ite. Ite above Pop_{1-2}. Pop_{1-2} on dorsal half, and Pop_3 separated by the former two, on ventral half of preopercular margin. Tea and Tep present.

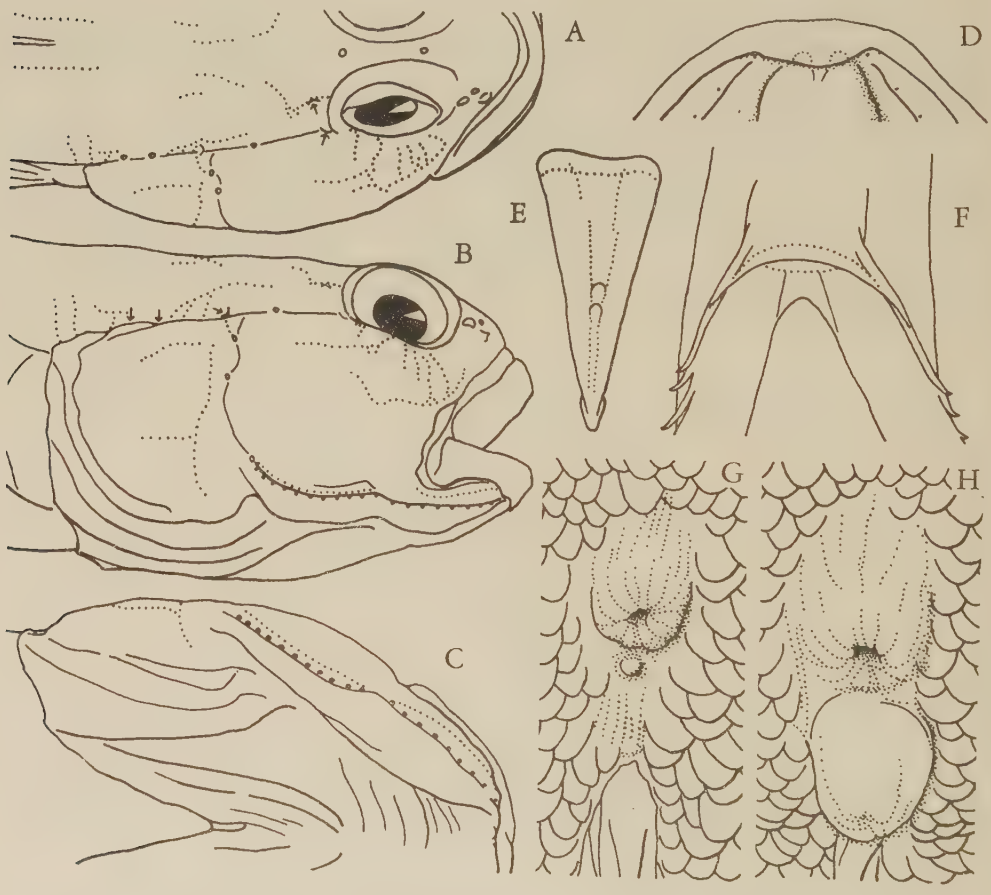


Fig. 2. *Amblyeleotris japonicus*, sp. nov. A–C, canal pore and pit-line disposition in dorsal (A), lateral (B), and ventral (C) views. Pit-line disposition is not exactly itself on the specimen, but partly diagrammatical one; dots do not correspond with each pit-organ. D, a pair of pockets opening behind chin under the mental fold, in ventral view. E, glossohyal bone. F, pelvic fin, especially on basal half. G–H, anal papilla in male (G) and female (H). Drawn from specimens of 83.8 mm. long, type (A–D, F, and G), 74.0 mm. long, male (E), and 83.8 mm. long, female (H).

Head and nape naked backward to between predorsal grooves, as well as on cheek and opercles. Scales on anterior half of body very small, rather round, cycloid, those on the rest of body and tail, ctenoid, enlarged gradually towards caudal peduncle, where they are of developed hexagoal shape. Eleven scales in a transverse row, measured from the last ray of second dorsal fin down to middle of caudal peduncle.

First dorsal fin higher than body, 1.4 height of body, third one longest. Second dorsal and anal fins lower than body, posteriorly pointed. Pectoral fin shorter than head, 0.8 head, more or less pointed by middle rays. Pelvic fin, with weak low membranes at base both anteriorly and posteriorly, sharp by longest innermost rays, shorter than head, 0.9 head. Caudal fin rather pointed, longer than head, 1.4 head.

Colour in formalin whitish gray on body, with four soft broad dusky cross bands, and a dusky spot on base of caudual fin. Head darker; dusky cross band on nape and operculum; upper lip, snout and interorbital space darker. Chin dusky; longitudinal dusky wavy line runs on each side of ventral surface across branchiostegeal membrane from behind chin to lower base of pectoral; its inner margin whitish. First dorsal fin dusky on basal half, with whitish spots on intermembranes; pale on distal half. Second dorsal fin dusky, a white longitudinal band at a fourth of height from base, and two or three elliptical small dusky spots on distal margin of each intermembrane. Anal fin blackish gray, except its palish narrow basal band with narrow dusky line along inner margin of brackish half. Caudal fin whitish gray, with radical dusky soft bands from dorsal and ventral base of caudal peduncle; dorsal half with some dusky small spots on distal margin, rest of which darker. Pelvic fin dusky on intermembrane. Pectoral fin palish.

Here described from the type specimen of 83.8 mm. long, male, secured July 15, 1954, from Kan-no-se (Tide-pool), Kagoshima Bay, Kagoshima Pref., by Mr. Kantaro NAKAHARA. Also seen four other specimens taken at the same time: 84.8, 83.8, and 65.6 mm. long, female, and 84.0 mm. long, male.

The genus *Amblyeleotris* BLEEKER, 1874, has included two species: the type species, *A. periophthalmus* (BLEEKER, 1855)[1], and next the other species, *A.* (*Andameleotris*) *raoi* HERRE, 1941. The second one, however, is separated afterwards (HERRE 1945: 2) from the genus in question and set near to the genus *Ptereleotris*, as *Andameleotris raoi*. The genus here mentioned has actually stood only on the rare species,[2] *A. periophthalmus*.

1) BLEEKER made thereafter some mentions of the genus and species, 1857: 465, 1874a: 374, 1877: 101. WEBER and DE BEAUFORT (1911: 59) cited the BLEEKER's paper No. 453 (1874b: 307) for the genus concerned, which, however "does not occur on this page or elsewhere in this paper." (acc. BOESEMAN's corresp.)

2) This species is said only known from two records: BLEEKER, 1953: 477, 75 mm., Batavia, now Djakarta (in sea), Java; WEBER, 1913: 450, 75 mm., Nalahia, Nusa Laut, Molukkas. GÜNTHER (1861: 124) and BORSIERI (1904: 207), basing surely on BLEEKER (1858: 212), added a record at Goram to those of the above localities. They seem to misread, as we see BLEEKER (1877: 102), and besides WEBER and DE BEAUFORT (1911: 59, 166, 168), and HERRE (1954: 370-371).

This genus has been related to some more or less unreliable records: *Eleotris periophthalmus* of KAROLI (1882: 167, Borneo) and BORSIERI (1904; 207, Nocra, Dahlach Is., Red Sea, Eritrea), and *Valenciennesia notophthalmus* BLEEKER, 1873 (China, from a sketch). According to KOUMANS (1953: 341), the identification of the former two may be doubtful. The last one was referred by BLEEKER (1874a: 374, 1875: 106, 1877: 100) as a probable another species in the genus in question.

The new taxon here described, *A. japonicus*, is very near to the type species, but it may be distinguished by: smaller eye (4.5–4.9 in head), longer snout (0.7 eyediameter), shorter maxillery (extending at most to below posterior margin of pupil), scales superior in number (28–33 in transverse row), and longer caudal fin (1.3–1.4 head), from the latter (4, 0.5, to below posterior margin of eye, 22, and 1, respectively), if overlooked their remarkable difference in coloration, as noted HERRE (1954: 346). Besides, it has no resemblance to the rest, at least except of *E. periophthalmus* of KAROLI, upon which I have been able to get in no sufficient information.

The sensory line system in Eleotridae is at first studied by HARRY (1948), especially on the genera, *Bunaka*, *Odontobutis*, and *Ophiocara*. The character of "a well defined furrow above the eye" is said to correspond to the divsion of BLEEKER's subfamily groups of *Butii* and *Eleotrii*. The genus *Amblyeleotris* may be then subject to Butinae, under a form as seen in *Odontobutis*, though no resemblance in most of the system arrangement between them.

Counts and dimensions (mm.) of *Amblyeleotris japonicus*, sp. nov.

Body length	84.8	83.8	65.6	83.8	74.0
Total length	115.9	113.4	90.4	115.0	99 5
Dorsal fins	VI–I–13	VI–I–13	VI–I–13	VI–I–13	VI–I–13
Anal fin	I–14	I–14	I–14	I–13	I–13
Pectoral fin	19	18	18	18	19
Ll	82	81	82	82	83
Ltr	32	31	28	32	33
Gill rakers	5+1+8	4+1+7	4+1+8	5+1+8	4+1+9
Canines	1–2|2–1 / 4 | 4	3–1|1–3 / 3 | 3	1–1|1–1 / 1–1|2–1	3 |1–1 / 2–1| 3	1–1| 1–1 / 2–1 |1–1–1
Sex	♀	♀	♀	♂	♂
Body height	15.5	15.1	11.0	13.6	11.6
Head length	23.3	22.5	17.6	22.2	20.2
Eye diameter	5.1	5.0	3.6	4.6	3.8
Interorbital space	1.5	1.2	1.0	2.2	1.2
Snout length	3.5	3.4	2.4	3.2	2.8
1st dorsal fin (longest)	19.1	19.0	12.6	19.6	15.2
Pelvic fin	21.0	20.0	12.3	19.4	18.7
Pectoral fin	19.0	17.6	15.9	18.5	16.3
Caudal peduncle length	12.0	11.1	11.2	12.0	11.7
Caudal peduncle height	8.6	8.1	6.0	8.5	7.0

2. ***Ctenogobius dotui***, sp. nov.
New Japanese name: Shiranuhi-haze.

Dorsal fin VI–I–10, anal fin I–12, pectoral fin 16. Scales 26 in a rateral row, 7 in a transverse row.

Body elongate, compressed, height 7.5 in length, 9.4 in total length. Head moderately depressed, 3.5 in length, 4.4 in total length. Eye 4.7 in head, interorbital narrow, 0.2 eyediameter. Anterior nostril with short tube, opens a little above anterior margin of preorbital region; posterior nostril without tube just behind nasal rising, and along anterior margin of orbit; nasal sac protrudes between nostrils and surrounded by furrows dorsally and ventrally. Snout very short, 0.7 eyediameter; tip pointed, inferior to level of lower margin of eye. Mouth moderate, rather oblique. Lower jaw a little prominent. Teeth simply pointed, curved inwards, on teeth band; on upper jaw in 3 rows, outer and inner rows more developed; on lower jaw in 2 rows anteriorly, in one row posteriorly, outer one most developed. Tongue rather truncate than rounded, smooth on dorsal surface, well separated from mouth floor. Glossohyal bone of developed fan-shape, more or less rounded on distal margin. Gill-opening moderate, lateral; isthmus wide, branchiostegeal membranes well separated. Gill rakers short, pointed at larger ones, 2+1+10 in number. Anal papilla well distinguished in shape between both sexes, slender and pointed in male, short and blunt in female. Caudal peduncle compressed, 2.7 in head, height 1.4 in its length.

Canal pores fully developed, all without tube opening. Na opens above posterior nostril. Itoa and Itop present, both single. Itop rather behind interorqital space. Sot seen. Ota and Otp occur, the latter well backwards, near to Pop_1. Ite present, however, therefore backwards at middle of Otp and Tea. Pop_{1-3} open on dorsal half of operculum by same distance. Tea and Tep both seen. Canal structure superficial, well distinctive by stain injection.

Head and nape naked backwards to predorsal region between longitudinal grooves. Scales on body large, ctenoid with well degenerated several spines on both trunk and tail; those on tail hexagonal with rounded blunt angles. Breast and belly scales cycloid.

First dorsal fin higher than body, second ray longest. Second dorsal fin about as high as body. Anal fin a little lower than body. Both second dorsal and anal fins pointed posteriorly. Pectoral fin shorter than head, elongated and pointed at middle rays. Pelvic fin a little shorter than head, elongate, elliptical in disc, extends to between insertions of first and second anal soft rays; basal membrane lower, fringed regularly on distal margin. Caudal fin oblong, rounded at end, shorter than head.

Colour in formalin pale brownish gray on both head and body. Dorsal and lateral scales bear minute dark spots on distal margin. On lateral median line of body some 10 not so distinct dusky spots, last one of which is on caudal base. Head furnished densely with small dusky spots. Ventral surface of head darker. First dorsal fin dusky, first ray darker. Second dorsal fin darker on distal half than basal one, which bears three dusky spots on each fin ray. Anal fin darker on intermembrane, especially on its distal half. Caudal fin dusky,

darker posteriorly, except of narrow pale margin. Pectoral fin pale. Pelvic fin dusky. As for the female, it differs only by a black spot at tip of first dorsal fin between fifth and sixth rays, and paler pelvic and caudal fins.

Here described the specimen of 30.0 mm. long, male, type, secured August 17, 1951, from estuary (brackish, dry-beach, Ariake-no-Umi) of the Rokkaku River, Saga Pref. Also seen four other specimens taken at the same time; 29.0 and 27.2 mm. long, female, 28.2 and 26.4 mm. long, male.

Dotui, dedicated to Mr. Yosie DÔTU, ardent contributor to the goby life-history, who offered me the interesting samples here presented.

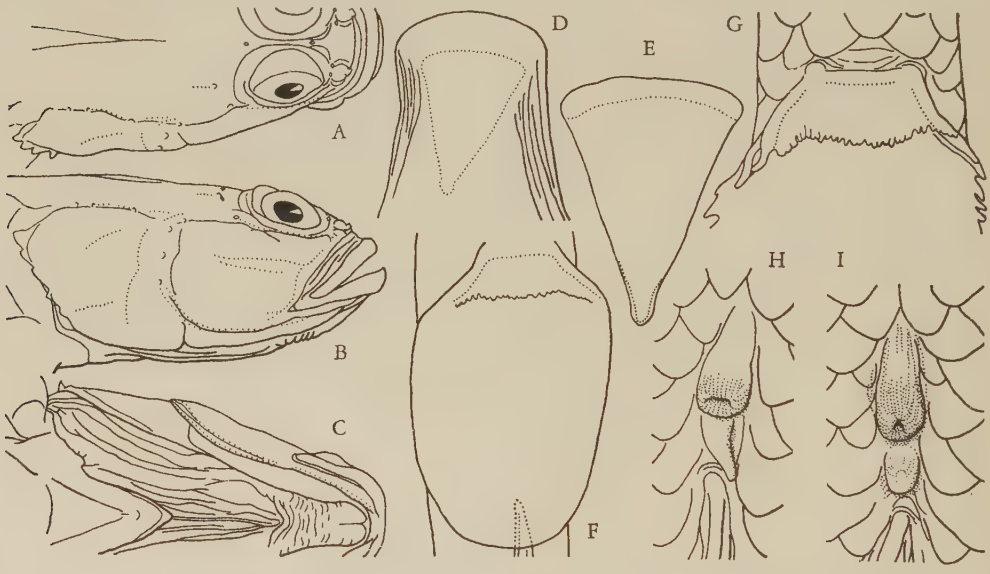


Fig. 3. *Ctenogobius dotui*, sp. nov. A–C, canal pore and pit-line disposition in dorsal (A), lateral (B), and ventral (C) views. See remarks on pit-lines in Fig. 1. D, tongue in relation to glossohyal bone (dorsal view). E, glossohyal bone. F–G, pelvic fin: diagrammatical disc form (F), and basal half in detail (G). H–I, anal papilla in male (H) and female (I). Drawn from specimens of 28.2 mm. long, male (A–C, F, G), 30.0 mm. long, type (D, E, H), and 29.0 mm. long, female (I).

The genus *Ctenogobius* GILL, 1858, has been under discussions by workers for its delimination. As the author of this genus "imperfectly defined" it, and there was not "a satisfactory limitation of the genus," before HERRE (1933), who offered a considerable suggestion to the matter, it causes some confusion among this genus and such genera as *Rhinogobius* GILL, 1859, and *Acentrogobius* BLEEKER, 1874. (See JORDAN and SNYDER, 1901: 54, JORDAN and HUBBS 1925: 305, HERRE 1927: 176, 177, 1933a: 265, 1945: 6, 1953: 742, 762, KOUMANS 1931: 88, 89, 1953: 176, e. g.)

The canal pore disposition will more or less through light on the matter. Comparing the disposition among the above three genera, based on our specimens of: *Rhinogobius similis* (nec GILL) JORDAN et SNYDER, 50.5 mm. long, no date,

Otsu, Shiga Pref., collected by Mr. Morizumi NAKAMURA, *Acentrogobius campbelli* (JORDAN et SNYDER), 94.7 mm. long, no date, Kominato, Chiba Pref., collected by Mr. Syûya NAKAMURA, and the present species, their relationship appears to be represented principally by Itoa. (Table 4.) The pore Itoa is seen single in *Ctenogobius* and *Acentrogobius*, and in a pair in *Rhinogobius*. This genus, as well as *Ctenogobius*, is furnished as generic character with very narrow interorbital space, as already known, of "about 1/4," and "somewhat less than 1/2" of eye-diameter respectively (KOUMANS 1931: 84, 1953: 176). The pairing of Itoa, and probably of Itop, therefore, seem to have no connection with the width of interorbital space. After all, it is very interesting that, so far as shown in my small number of samples, the relationship seen in the canal pore disposition among these genera apparently corresponds to, or rather serves to confirm, the recent results saying that *Ctenogobius* is separable to *Rhinogobius*, as well as to *Acentrogobius*.

Table 4. Canal pore disposition in *R. similis*, *A. campbelli*, and *C. dotui* (from Table 1). See remarks on Table 6.[1]

Canal pores	Na	Itoa	Itop	Sot	Ota	Otp	Ite	Pop_1	Pop_2	Pop_3	Tea	Tep
Rhinogobius similis	1	1	(1)	1	1	1	1	1	1	1	1	1
Acentrogobius campbelli	1	(1)	(1)	1	1	1	1	1	1	1	1	1
Ctenogobius dotui	1	(1)	(1)	1	1	1	1	1	1	1	1	1

According to recent definitions by HERRE (1933a) or KOUMANS (1941: 265, 1953b: 14–19, Key, 176), the species here erected may be agreable to be put in the said genus, and it appears very near to *C. nuchipunctatus* HERRE, (1933b) 1934, or *C. villadolidi* HERRE, 1936. Their principal resemblances and differences are shown in Table 5. The final generic decision, however, will be after more aculate ascertainment of the generic character of the species, *C. fasciatus* GILL, 1858, information of which have been given little to us; for instance, it may be critical if the basal membrane of pelvic fin is fringed or not on free margin in the type species, as seen between *Acanthogobius* and its related (TOMIYAMA 1936: 84, MATSUBARA 1955: 824).

Table 5. Relationships among *C. dotui* and related on some principal counts and proportions

Ctenogobius	*dotui*	*nuchipunctatus*	*villadolidi*
Dorsal soft rays	10	11	12
Anal soft rays	12	12–13	12–13
LI	25–26	26–27	(27–28)+1
Ltr	7	9	8
Body height in length	6.8–7.5	5.8	6.4–6.7
Eye in head	4.5–5.0	4	3.9–4.1
Black spots on sides	10	4	5

Counts and dimensions (mm.) of *Ctenogobius dotui*. sp. nov.

Body length	29.0	27.2	30.0	28.2	26.4
Total length	35.2	33.2	37.4	36.7	33.6
Dorsal fins	VI–I–10	VI–I–10	VI–I–10	VI–I–10	VI–I–10
Anal fin	I–12	I–12	I–12	I–12	I–12
Pectoral fin	16	16	16	17	17
Ll	25	26	26	25	25
Ltr	7	7	7	7	7
Gill rakers	—	—	2+1+10	—	—
Sex	♀	♀	♂	♂	♂
Body height	4.0	4.0	4.0	4.0	3.6
Head length	8.1	8.0	8.5	8.3	8.0
Eye diameter	1.8	1.7	1.8	1.8	1.6
Interorbital space	0.3	0.3	0.3	0.3	0.3
Snout length	1.3	1.2	1.3	1.3	1.2
Maxillary length	2 3	2.3	2.7	2.5	2.5
1st dorsal fin (longest)	5.0	4.7	4.9	4.2	4.7
Pelvic fin	7.2	7.1	8.0	6.8	7.2
Pectoral fin	6.5	6.2	7.5	6.2	6.4
Caudal peduncle length	3.0	3.2	3.2	3.0	3.0
Caudal peduncle height	2.3	2.4	2.8	2.4	2.4

3. ***Callogobius shunkan***, sp. nov.

New Japanese name: Shunkan-haze.

Dorsal fin VI–I–10, anal fin I–8, pectral fin 17. Scales 32 (??33) in a lateral row, 11 in a transverse row.

Body elongate, anteriorly cylindrical, posteriorly compressed; height 5.6 in length, 7.2 in total length. Head moderately depressed, 3.4 in length, 4.4 in total length. A pair of minute antennary papilla behind interorbital space, as seen on *Callogobius hasselti*[1]. Eye 5.0 in head; interorbital narrow, 0.5 eyediameter. Nostrils with developed tube opening; anterior one a little above upper lip; posterior one behind it at middle of snout. Snout obtuse, 1.6 eyediameter; tip inferior to level of lower margin of eye. Mouth rather small, oblique to vertical; lower jaw prominent. Teeth of outer row enlarged to be of canine-like; those of inner row of villiform. Tongue emarginate at tip, rather smooth anteriorly, rough posteriorly with tubercles on dorsal surface; moderately separated from mouth floor. Glossohyal of well developed fan-shape, rounded on distal margin, except of smoothly emarginate middle tip. Gill opening lateral, not very wide; isthmus broad. Gill-rakers compressed, slender, 3+1+8 in number. Dermal folds on head with pit-line on distal margin occur on snout, occiput, cheek and opercle; a pair of three transverse folds behind chin on each side; the same folds on body. Anal papilla well developed to be probably distinguishable in shape

1) 57.4 mm., July 30, 1950, Kan-no-Se, Kagoshima Pref., collected by Mr. Y. DÔTU.

between both sexes; slender, pointed in male, exceeds anal spine at tip. Caudal peduncle compressed, 1.1 in head; height 1.9 in its length.

Canal pores fully developed; most of them with tube opening. Na opens by tube, just before interorbital space. Itoa and Itop both single, with more or less long and wide tube. Sot protrudes, opening laterally. Ota opens by small tube. Otp below Sot, protrudes, with antero-lateral opening. Ite opens laterally by very low tube or ridge, proceeds to near Otp. Pop_{1-3} opens by small tube, all lining vertically on middle one third of preopercular margin by same space. Pop_3 just below end of upper *v.l.* (Lekander 1949, or posterior hc_1 of Stensiö 1947: 80, Fig. 21, B). Tea and Tep protrudes as well as their connection, opening anteriorly and posteriorly, respectively.

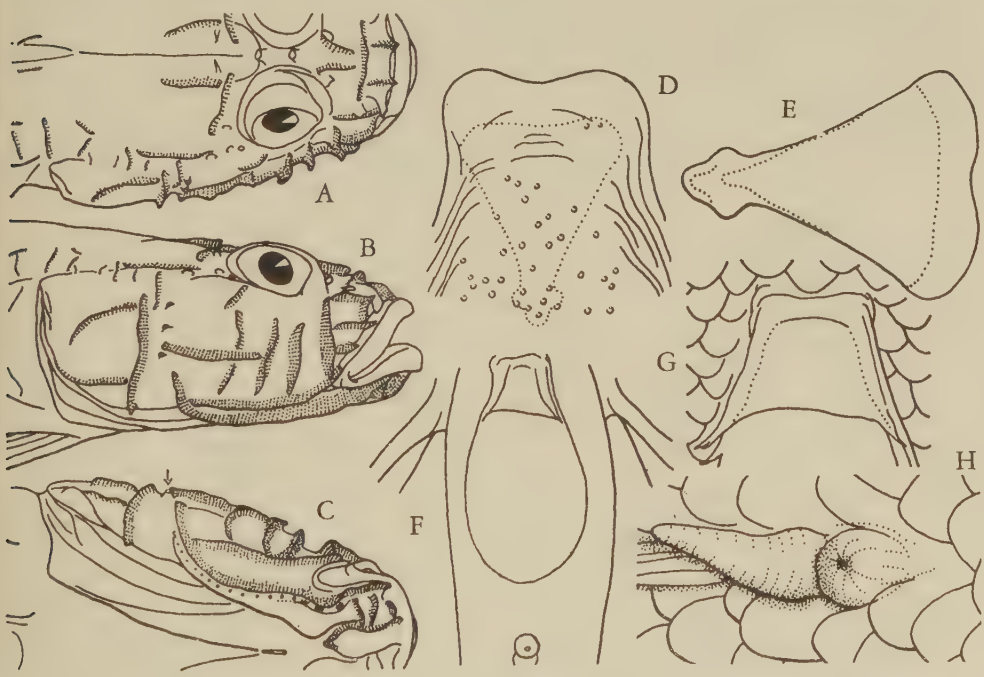


Fig. 4. *Callogobius shunkan*, sp. nov., type. A–C, canal pore and pit-line disposition in dorsal (A), lateral (B), and ventral (C) views. Pit-lines are on distal margin of fleshy or dermal folds; dots do not represent pit organs but structure of the folds. D, tongue in relation to glossohyal bone (dorsal view). E, glossohyal bone. F–G, pelvic fin: diagrammatical disc form (F), and basal half in detail (G). H, anal papilla.

Scales elusive on head and body; those of body ctenoid posteriorly, cycloid anteriorly. Head scaled above behind eye; predorsal ?8; opercle scaled.

First dorsal fin higher than body; first spine longest. Second dorsal and anal fins lower than first dorsal fin, pointed posteriorly. Pectoral fin rather oblong, a little shorter than head. Pelvic fin apparently shorter than head, elliptical in disc; basal membrane developed, deep, smooth on its free margin. Caudal fin oblong, about as long as head.

Colour in formalin reddish brown, which is faded dorsally and ventrally. Folds on head and body dark brown. First and second dorsal, pectoral, and caudal fins with more or less many small light spots, on irregular longitudinal (dorsal) or transverse (the rest) rows. Anal and pelvic fins both dusky.

Shunkan, from Shunkan Sôzu, sub-bishop in the Buddhism (1142–79), a tragic hero in a Japanese historical literature, the "Heike-monogatari."

Here described from the type specimen of 59.0 mm. long, male, secured July 31–August 3, 1943, by Mr. Sadahiko IMAI, from Matsugaura, Chiran, Kagoshima Pref. Also seen another specimen of 49.0 mm. long, male, taken at the same time. More or less malpreserved.

The new species here mentioned is very near to *Callogobius snelliusi* KOUMANS, 1953 (KOUMANS 1953a: 248, 1953b: 102–103). The new taxon, however, having 10 dorsal softray, 8 anal softrays, lower body height (5.6 in length), smaller eye (5.0 in head), longer snout (1.6 eye diameter), and emarginate tongue at tip, may be distinguished to the latter (8, 6, 4, 3, a little shorter than eye, and rounded, respectively).

Counts and dimensions (mm.) of *Callogobius shunkan*, sp. nov.

Body length	59.0	49.0
Total length	76.0	62.4
Dorsal fins	VI–I–10	VI–I–10
Anal fin	I–8	I–8
Pectoral fin	17	17
Ll	32 (or ??33)	31
Ltr	11	10
Predorsal scales	?8	?8
Gill rakers	3+1+8	—
Sex	♂	♂
Body height	10.5	8.7
Head length	17.2	15.4
Eye diameter	3.4	3.2
Interorbital space	1.7	1.2
Snout length	5.3	5.0
Maxillary length	4.6	3.6
1st dorsal fin (longest)	15.0	13.0
Pelvic fin	12.3	11.5
Pectoral fin	15.2	14.7
Caudal peduncle length	15.0	11.8
Caudal peduncle height	7.7	6.7

4. ***Stigmatogobius minutus***, sp. nov.
New Japanese name: Ichimatsu-haze.

Dorsal fin VI–I–6, anal fin I–6, pectoral fin 17. Scales 26 in a lateral row, 7 in a transverse row.

Body rather elongate, compressed; height 4.0 in length, 5.0 in total length. Head moderately compressed, 3.1 in length, 3.8 in total length. Eye 4.2 in head; interorbital 0.5 eyediameter. Anterior nostril with tube opening, which prolonged forewards beyond upper margin of upper lip; posterior one without tube opening, above anterior one and before centre of eye. Snout obtuse, shorter than eye, 0.8 eyediameter; tip inferior to level of lower margin of eye. Mouth more or less oblique. Jaws subequal. Maxillary extends to below hinder margin of eye in male, below middle of eye in female. Teeth minute, simple, more or less curevd inwards; on upper jaw in one row, some front teeth larger, hook-like as curved well at tip, lateral ones smaller; on lower jaw, front teeth in tow rows, smaller, lateral ones in one row, at least some of them larger, hook-like. Tongue rough on dorsal surface with tubercles, which occur laterally in two longitudinal bands, moderately separated from mouth floor at middle. Glossohyal bone of fan-shape, rather narrow, truncated on distal margin. Gill-opening lateral, moderate, isthmus broad. Gill-rakers not developed, shorter and smooth, 2+1+6 in number. Anal papilla equally small low cone-like, and not so distinguishable between both sexes, but by more or less sharper tip in male. Caudal peduncle about as long as head, 1.0 in head, 2.2 in its length.

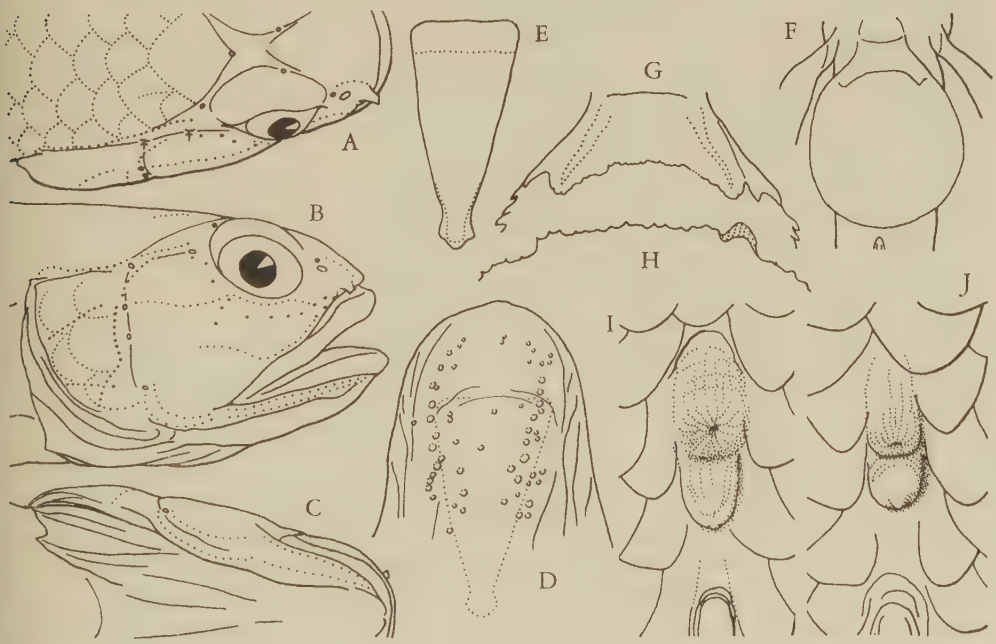


Fig. 5. *Stigmatogobius minutus*, sp. nov. A–C, canal pore and pit-line disposition in dorsal (A), lateral (B), and ventral (C) views. See remarks on pit-lines in Fig. 1. Squamation on nuchal (A) and opercular (B) regions is represented by smaller dots. D, tongue in relation to glossohyal bone (dorsal view). E, glossohal bone. F–H, pelvic fin: diagrammatical disc form (F), and basal half (G) and free margin of basal membrane (H) in detail. I–J, anal papilla in male (I) and female (J). Drawn from specimens of 26.2 mm. long, type (B, D–I) and 23.9 mm. long, female (A, C, J).

Canal pores all developed but Otp, Tea and Tep, which are closed together. Na just behind upper margin of posterior nostril. Itoa in a pair. Itop single, opens on foremost nupcial scaled area. Ota just behind orbit. Ite opens just above upper preopercular margin. Pop_{1-2} along middle of preopercular hind margin, Pop_3 separated from them, near to lower angle of preoperculum.

Head scaled above behind eye; predorsal scales 7, cycloid, hexagonal with round angles; foremost scale unpaired, larger. On operculum cycloid, and irregul larly rounded scales. All ctenoid ones on trunk and tail; those of tail longer hexagonal, anterior angle indistinct, with well developed spines on hinder two margins.

First dorsal fin possibly higher than body in maturated male, or else about as high as body in both sexes, when middle rays longest. Second dorsal and anal fins lower than body, pointed posteriorly. Pectoral fin a little shorter than head, moderately oblong, middle rays longest. Pelvic fins shorter than head covering anus by its free margin, which is rounded; basal membrane lower, rough on distal margin. Caudal fin shorter than head, rounded.

Colour in formalin yellowish, scales dusky on posterior margin. Brownish gray cross bands on dorsal half of head and body, narrower one both on nape and predorsal, broadest one on first dorsal base, and one each on anterior and posterior base of second dorsal fin. Five dark spots longitudinally on lateral median line, two dark spots transversely at the caudal fin base. Below median spots 6 dark spots, foremost two on side, the rest four on ventral median line. On head two dark vertical lines under eye, Y-shape line vertically on preoperclum, a large dark spot on operculum, under which two transverse line on subopercular region. First dorsal fin pale, except of a few dark spots on rays, and larger spots on margin of middle rays and base of last rays. Second dorsal pale, some three lines of spots on rays, distal margin dusky. Caudal fin with some vertical lines of spots on rays anteriorly, dusky posteriorly. Pectoral fin pale. Pelvic fin more or less dusky, innermost rays darker.

Here described from the specimen of 26.2 mm. long, male, type, taken June 1, 1949, from Tomari (estuary), Satsuma Pen., Kagoshima Pref. Also seen other three specimens taken December 18, 1952, from Hitotsuba Firth, Miyazaki Pref., 23.9 and 21.5 mm. long, female, 21.7 mm. long, male., and some other ones from both localities.

The species here erected is very near to *Stigmatogobius romeri* (WEBER, 1911), and less to *S. isognathus* BLEEKER, 1878. The new taxon having subequal jaws, maxillary extending to below hind margin of eye in male, uniserial teeth except in front of lower jaw, and a pair of distinct Itoa, differs from both *S. romeri* (lower jaw prominent, maxillary extends to behind eye in male, front teeth in several rows on both jaws, and a pair of "indistinct" Itoa[1]), and *S. isognathus* (jaws subequal, maxillary to below anterior margin of pupil, teeth in more or less some rows on both jaws, and no Itoa[1]). The coloration of the

1) As for these species, KOUMANS (1953b) described "open pores in interorbital....," (p. 114) or "no pore in interorbital," (p. 116). He means apparently the pore Itoa, here mentioned, by "pore in interorbital," and therefore, Itoa in a pair by "pores," because these descriptions are followed by those of single "open-pore behind eye," which means Itop accordingly. I wonder, however, if *S. isognathus* has no Itoa or not, which may be left in further scrutiny.

species in question agrees mostly with *S. romeri*, and not with *S. isognathus*, which is more simple in this point of view than the formers.

Counts and dimensions (mm.) of *Stigmatogobius minutus*, sp. nov.

Body length	23.9	21.5	26.2	21.7
Total length	30.0	27.7	32.3	27.8
Dorsal fins	VI–I–7	VI–I–7	VI–I–6	VI–I–7
Anal fin	I–6	I–6	I–6	I–6
Pectoral fin	16	17	17	16
Ll	26	26	26	26
Ltr	7	7	7	7
Predorsal scales	7	7	7	7
Gill rakers	2+1+6	—	2+1+6	—
Sex	♀	♀	♂	♂
Body height	5.5	5.1	6.5	5.0
Head length	7.5	6.9	8.4	7.3
Eye diameter	1.8	1.7	2.0	1.6
Interorbital space	1.0	1.0	1.1	0.8
Snout length	1.6	1.5	1.6	1.5
1st dorsal fin (longest)	4.2	4.4	7.3	4.8
Pelvic fin	5.8	5.4	6.5	5.8
Pectoral fin	6.3	6.4	7.3	6.9
Caudal peduncle length	7.0	6.5	8.3	6.5
Caudal peduncle height	3.2	3.1	3.8	3.0

Paleatogobius, gen. nov.

Body elongate, compressed, covered with very minute ctenoid scales along lateral mid-line on caudal peduncle. Head a little depressed, naked. Interorbital narrow, about half eyediameter. Mouth horizontal. Maxillary very prolonged, lower jaw a little prominent. Teeth minute, pointed, no canine. Tongue notched, or incised at tip. Glossohyal bone of Y-shape, with expanded wings. (Type L). On each side behind chin a short fleshy barbel prolonged backwards. First dorsal fin with six rays. Second dorsal fin I–10/11. Pelvic fins completely united, basal membrane developed, somewhat rough on distal margin. Caudal fin rounded. Canal pores: Na, no Itoa, and paired Itop present. (Type V.) A pair of pit-organs in a ditch just behind chin, and before barbels.

Type species: *Paleatogobius uchidai*, gen. et sp. nov.

Paleatogobius, subst. masc., *Paleato-*, having barbels, from *Palea*, wattle or barbel, which are seen behind chin.

The genus here described as new has a good resembrance with *Chaenogobius* GILL, 1862, principally by notched tongue and diverged glossohyal bone, canal pore disposition, especially no Itoa and paired Itop in interorbital space, and also no Tea nor Tep, and the pit-organs occurring in a ditch behind chin, but differs

from the latter genus by a pair of barbel behind chin, as well as by occurrence of Pop_{1-3} (no Pop_{1-3} in *Chaenogobius*), expanded wings of glossohyal bone (spaturate wings in *Chaenogobius*). The characteristics of Pop_{1-3}, and glossohyal wings, I suppose, are acceptable as the generic characters by which, also, are separable these two genera. As for the mental barbels in a pair, the genus *Paleatogobius* may be said to be related to *Illana* SMITH and SEALE, 1906, rather than to *Synechogobius* GILL, 1862. The new taxon in question, however, distinguished to *Illana*, as well as to the other, because of principally the shape of barbels, and squamation for instance.

5. ***Paleatogobius uchidai***, sp. nov.

New Japanese name: Chikuzen-haze.

Dorsal fin VI–I–11, anal fin I–10, pectoral fin 19.

Body elongate, compressed, height 6.4 in length, 7.8 in total length. Head moderately depressed, 3.8 in length, 4.6 in total length. Eye 4.2 in head, interorbital 0.6 eyediameter. Anterior nostril with short tube opening, found on front margin of preorbital region, posterior one with no tube, but erected ridge. Nasal sac somewhat protrudes. Snout obtuse, short, 1.1 eyediameter; tip before lower margin of eye. Mouth rather horizontal than oblique; lower jaw a little prominent, well convex at tip in lower profil. Maxillary extended nearly to lower angle of preoperculum in both sexes, 1.6 in head. A small ditch just behind chin, in which occurs a pair of pit-organs. A pair of barbels follows it, points backwards, with a few small processes in row just before its base. No teeth on upper jaw, single pointed teeth band on anterior half of lower jaw, in 2–3 rows anteriorly, in one row posteriorly; posterior teeth a little larger than anterior ones. Tongue smooth, with deeper notch or incision at tip, separated moderately from mouth floor at middle. Glossohyal bone of Y-shape, with well expanded wings, which is prolonged forewards. Gill-opening lateral, moderate; branchiostegeal membrane well separated. Gill-rakers well developed, slender, compressed, 4+1+10 in number. Anal papilla rather difficult to be distinguished in shape between both sexes, a little longer in female than in male. Caudal peduncle slender, 1.4 in head, height 2.3 in its length.

Canal pores rather degenerated, half of them omitted. Na opens before anterior end of interorbital side margin. Itoa not seen. Itop present in a pair. Sot and Ota closed. Otp opens just behind orbit. Ite, Tea and Tep all absent. Pop_{1-3} open in dorsal half of preopercular margin by same space.

Scales minute, cycloid and ctenoid, occur under skin along lateral mid-line on caudal peduncle as in *Astrabe cosmurus* (JORDAN et SYNDER).

First dorsal fin lower than body, middle rays longest. Second dorsal and anal fins as high as body. Caudal fin shorter than head. Pectoral fin rounded, oblong, shorter than caudal fin. Pelvic fin oblong, about as long as caudal fin, rounded at tip; basal membrane developed, somwhat rough on distal margin.

Colour in formalin brownish gray, dorsal half darker, with vermiform blotches, which are faded on ventral half. Dusky blotches on median line of the side form cross bands anteriorly and spots posteriorly.

Uchidai, dedicated to Prof. Keitaro UCHIDA at Kyushu University.

Here described by the type specimen of 25.5 mm. long, male, catched May 17, 1948, from firth of the Kanakuzu River, Fukuoka, Fukuoka Pref. Also seen five other specimens of: 30.0 mm. long, female, 25.0 mm. long, male, and 26.4, 25.5, and 23.0 mm. long, sex unknown.

The new taxon in question is very similar to *Chaenogobius murorana* (JORDAN et SNYDER), except of having a pair of barbels, and small size of body, as well as the pit-organs occurring behind chin, of which it is alike to *Chaenogobius cstanea* in shape. The mental pit-organs in a ditch will be mentioned below, in the discussion of the next species.

Fig. 6. *Paleatogobius uchidai*, gen. et sp. nov. A–C, canal pore and pit-line disposition in dorsal (A), lateral (B) and ventral (C) views. See remarks on pit-lines in Fig. 1. D, tongue in relation to glossohyal bone (dorsal view). E, glossohyal bone. F, ditch and barbels behind chin. G–H, pelvic fin: diagrammatical disc form (G) and basal half in detail (H). I–J, scales on tail, ca×55; no ridges or circuli drawn. K–L, anal papilla in male (K) and female (L). Drawn from specimens of 25.5 mm. long, type (A–C, F–H, K) and 30.0 mm. long, female (D, E, I, J, L).

Counts and dimensions (mm.) of *Paleatogobius uchidai*, gen. et sp. nov.

Body length	30.0	26.4	25.5	25.5	25.0	23.0
Total length	36.8	32.0	31.0	31.0	30.5	28.2
Dorsal fins	VI–I–11	VI–I–11	VI–I–11	VI–I–11	VI–I–10	VI–I–10
Anal fin	I–11	I–10	I–10	I–9	I–9	I–10
Pectoral fin	19	19	19	19	19	19
Gill rakers	—	—	—	4+1+10	—	—
Sex	♀	—	♂	—	♂	—
Body height	4.5	4.1	4.0	4.0	4.0	3.4
Head length	8.4	7.0	7.0	7.0	6.7	6.3
Eye diameter	1.8	1.7	1.6	1.7	1.6	1.3
Interorbital space	1.0	1.1	1.0	0.9	0.9	0.7
Snout length	1.9	1.7	1.8	1.8	1.6	1.6
Maxillary length	5.3	4.5	4.5	4.4	4.2	4.0
1st dorsal fin (longest)	3.2	—	2.4	—	—	—
Pelvic fin	6.3	—	5.0	—	—	—
Pectoral fin	5.5	—	4.8	—	—	—
Caudal peduncle length	5.5	5.5	5.0	4.9	5.1	4.4
Caudal peduncle height	2.5	2.3	2.2	2.3	2.2	2.0

6. ***Chaenogobius scrobiculatus***, sp. nov.
New Japanese name: Kubo-haze.

Dorsal fin VI–I–10, anal fin I–9, pectoral fin 18.

Body slender, cylindrical anteriorly, compressed posteriorly, height 8.3 in length, 10.3 in total length. Head depressed, 3.5 in length, 4.3 in total length, preopercular region fleshly inflated. Eye 5.0 in head, interorbital narrow, 0.7 eye diameter. Anterior nostril with tube opening close to upper lip. Posterior one without tube, opens on a projection. Mouth inferior, horizontal, lower jaw plugged in upper jaw. Maxillary extends to below behind eye, 1.5 in head. No teeth on upper jaw, rather uniformal canine-like teeth band fully developed on lower jaw, in three or more rows in front, in one row laterally. Tongue rough with tubercles on dorsal surface, notched broadly at tip, separated moderately from mouth floor. Glossohyal bone of Y-shape; wings expansively diverged, each of them narrow, hardly broadened anteriorly. Gill-opening broad, somewhat exceeds ventrally, isthmus broad. Gill-rakers smooth, short, 3+1+7 in numebr. A small ditch occurs just behind chin, furnished with a pair of rather developed pit-organs in it. Anal papilla rather difficult to distinguished between both sexes, though fatter in female. Caudal peduncle, short and low, 1.5 in head, 2.3 in its length.

No canal pores occur but three pairs of Na, Itop, and Sot. Na opens at anterior end of interorbital margin (or groove).

Scales minute, embeded on body sides, cycloid anteriorly, ctenoid posteriorly. Caudal scales rather broadly rounded than pentagonal, with rather developed spines occurring in line on posterior margin.

First dorsal fin about as high as body, middle rays longest. Second dorsal and anal fins almost as high as body, middle rays longest. Pectoral fin oblong, shorter than head. Pelvic fin oblong, shorter than pectoral fin, elliptical in disc; basal membrane developed, rather smooth on its margin. Caudal fin oblong, shorter than head; middle rays rounded to truncate posteriorly.

Fig. 7. *Chaenogobius scrobiculatus*, sp. nov. A–C, canal pore and pit-line disposition in dorsal (A), lateral (B) and ventral (C) views. See remarks on pit-lines in Fig. 1. D, tongue in relation to glossohyal bone (dorsal view). E, glossohyal bone. F, ditch with pit organs in it behind chin. G–H, pelvic fin: diagrammatical disc form (G), and basal half in detail (H). I–J, scales on tail (I) and trunk (J), ca×50. K–L, anal papilla in male (K) and female (L). Drawn from specimens of 35.2 mm. long, type (A–C), 32.5 mm. long, male (D, E, I, J), 35.0 mm. long, female (F–H), 29.7 mm. long, male (K), and 31.8 mm. long, female (L).

Colour in formalin light brown, ventral half whitish; snout, interorbital groove, and cheek darker; many irregular narrow cross bands on body darker, distinct on ventral half. First and second dorsal, and caudal fins with darker spots form narrow longitudinal or cross lines, as well as pectoral fin, where spots are not distinct. Anal and pelvic fins dusky.

Scrobiculatus, having a small ditch, from the mental one, above mentioned.

Here described from the specimen of 35.2 mm. long, male, captured March 19, 1951, at the firth of the Muromi River, Fukuoka, Fukuoka Pref. Also seen other four specimens taken at the same time: 35.0, 32.5, and 31.8 mm. long, female, and 29.7 mm. long, male.

The new species has a good resemblance with *Chaenogobius cylindricus* TOMIYAMA (1936: 92, 55 mm. long), but differs from the latter by embeded lesser ctenoid scales (distinct 80 cycloid scales in lateral series in the latter), and prolonged maxillary of 1.5 in head (2 in head in the latter).

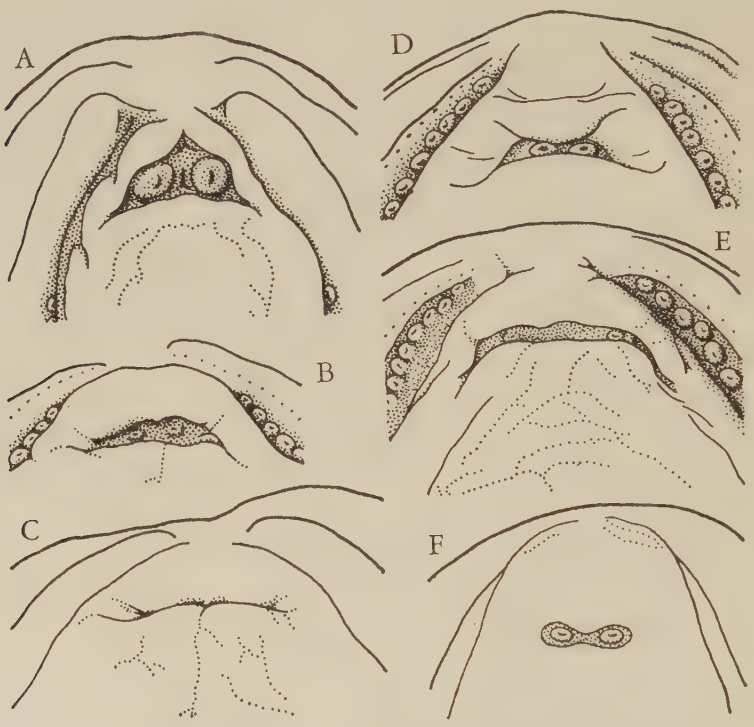


Fig. 8. Mental ditch with pit organs in it of some known *Chaenogobius* species. A, *C. castanea* (O'SHAUGHNESSY): ditch sharrow, pit organs developed. B–C, *C. urotaenia* (HILGENDORF): ditch deeper, but not open in normal condition (C), pit organs not distinct. D, *C. murorana* (JORDAN et SNYDER): ditch sharrow, pit organs developed. E, *C. isaza* TANAKA: ditch rather sharrow, pit organs not distinct. F, *C. heptacanthus* (HILGENDORF): ditch very sharrow, pit organs not developed, but distinct. Drawn from specimens of: A, 48.5, B–C, 71.0, D, 50.0, E, 47.0, and F, 48.5 mm. long.

As for the small ditch behind chin, it is interesting that all *Chaenogobius* species but *C. macrognathus*, and *C. cylindricus*[1], are found to be furnished with it, and that they are not equal in its size and form (Fig. 8, see remarks);

1) I can not examine the ditch on these two species. *C. macrognathus* (BLEEKER, 1860) must to be put in *Gymnogobius* BLEEKER, 1863, according to TARANETZ (1934), who regards the latter as the valid genus. (See also KOUMANS 1931: 52, MATSUBARA 1955: 838.)

The type specimen of *C. cylindricus* is said to be ruined at Hiroshima University Museum by the A-bomb raid. (Acc. Messrs. A. INABA and R. KATASHIMA's corresps.)

the most degenerated form is seen in *C. heptacanthus* (Hilgendorf), while the most developed one in the present new species, as shown in Figs. 7, F, and 8, F. The ditch is also seen in *Paleatogobius*, as mentioned already, and probably homolgous to mental pockets as seen in *Amblyeleotris*, above, which may be of much more primitive form. This organ is surely the generic character in these genera.

Counts and dimensions (mm.) of *Chaenogobius scrobiculatus*, sp. nov.

Body length	35.0	32.5	31.8	35.2	29.7
Total length	43.9	40.2	40.5	43.3	38.1
Dorsal fins	VI–1–10	VI–I–10	VI–I–10	VI–I–10	VI–I–11
Anal fin	I–9	I–8	I–9	I–9	I–10
Pectoral fin	18	18	18	18	18
Gill rakers	—	—	—	3+1+7	—
Sex	♀	♀	♀	♂	♂
Body height	5.4	4.2	4.2	4.2	3.5
Head length	10.0	8.6	9.2	10.0	9.0
Eye diameter	1.8	1.7	1.8	2.0	1.8
Interorbital space	1.4	1.1	1.2	1.4	1.2
Snout length	2.1	1.9	2.0	2.5	2.1
Maxillary length	6.2	5.4	5.5	6.5	5.9
1st dorsal fin (longest)	5.0	3.5	4.0	4.1	3.9
Pelvic fin	6.3	5.4	5.5	6.3	5.7
Pectoral fin	7.0	6.3	6.5	7.2	6.4
Caudal peduncle length	6.6	6.3	5.9	6.8	5.7
Caudal peduncle height	2.8	2.3	2.5	3.0	2.8

References

The papers with asterisk are not referred to directly.

Bleeker, Pieter

1853. Diagnostische beschrijvingen van nieuwe of weining bekende vischsoorten van Batavia. Tiental I–VI. Naturkundig Tijdschrift voor Nederlandsch-Indië 4: 451–516.

1857. Index descriptionum specierum piscium Bleekerianarum in voluminibus I ad XIX Diarii Societatis Scientiarum Indo-Batavae. ibid. 14: 447–486.

*1958. Bijdrage tot de kennis der vischfauna van den Goram-Archipel. ibid. 15: 197–218.

*1873. Mémoire sur la faune ichthyologique de Chine. Nederlandsche Dierkundige Vereeniging, Tijdschrift 4: 113–154.

1874a. Notice sur les genres *Amblyeleotris*, *Valenciennesia* et *Brachyeleotris*. Koninklijke Akademie van Wetenschappen, Verslagen en Mededeelingen, Afdeeling Natuurkunde, [2] 8: 372–376.

1874b. Esquisse d'un système naturel des Gobioïdes. Archieves Néerlandaises des Sciences Exactes et Naturelles 9: 289–331.

1875. Notice sur les Eleotriformes et description de trois espèces nouvelles. ibid. 10: 101–112.

1877. Révision des espèces insulindiennes de la sous-famille des Eleotriformes. Koninklijke Akademie van Wetenschappen, Verslagen en Mededeelingen, Afdeeling Natuurkunde, [2] 11: 13–110.

1878. Description des espèces insulindiennes du genre *Stigmatogobius*. ibib. 12: 199–208.

BORCEA, Iean

1933. Révision systématique et distribution géographique des Gobiidés de la Mer Noire et particulièrement des eaux roumaines. Université de Jassy, Annales Scientifiques 19 (1–4): 1–231, figs. 91, pls. 8.

BORSIERI, Clementia

1904. Contribuzione alla conscenza della fauna ittiologia della colonia Eritrea. Museo Civico di Storia Naturale "Giacomo Doria," Genova, Annali [3–1] 41:187–220.

DE BUEN, Fernando

*1923. *Gobius* de la peninsula Iberica y Baleares. Grupos *Lesueurii*, *Colonianus*, *Affinis* y *Minutus*. Instituto Español de Oceanografía, Memoria 3 (3): 117–266, figs. 170.

1930. Sur une collection de Gobiinae provenant du Maroc. Essai de synopsis des espèces de l'Europe. Société des Sciences Naturelles du Maroc, Bulletin 10 (7–9): 120–147, figs. 12.

CORDIER, Robert

1954. Le système nerveux central et les nerfs cérébro-spinaux. in Traité de Zoologie: Anatomie, Systématique, Biologie, 12. viii+202–332, figs. 63. Paris.

FAGE, Louis

1914. Sur le *Gobius minutus* PALLAS et quelques formes voisines. Société Zoologique de France, Bulletin 39 (7): 299-314, figs. 6.

1915a. Remarques sur l'évolution des Gobbiidae, accompagnées d'un synopsis des espèces européennes. Archieves de Zoologie Expérimentale et Générale 55, Notes et Revue 2: 17–34.

1915b. Sur quelques *Gobius* méditerranéens (*G. Kneri* STNDR., *G. elongatus* CANESTR., *G. niger* L.). Société Zoologique de France, Bulletin 40 (8–10): 164–175, figs. 5.

FOLLETT, W. I.

1955 (Not published). An unofficial interpretation of the International Rules of Zoological Nomenclature..... v+99.

FRASER-BRUNNER, A.

1949. A classification of the fishes of the family Myctophidae. Zoological Society of London, Proceedings 118 (4): 1019–1106, fis. 14, with many in key, pl 1.

GILL, Theodore N.

1858. Synopsis of the freshwater fishes of the western portion of the island of Trinidad, West Indies. Lyceum of Natural History, New York, Annales 6: 374–376 and 430.

*1860. Note on a collection of Japanese fishes made by Dr. J. MORROW. Academy of Natural Sciences of Philadelphia, Proceedings (1859): 144–150.

GOSLINE, William A.

1949. The sensory canals of the head in some cyprinodont fisnes, with particular reference to the genus *Funduls*. Museum of Zoology, University of Michigan, Occasional Papers 519: 1–18, fig. 1, pls. 2.

GÜNTHER, Albert

1861. Catalogue of the acanthopterygien fishes in the collection of the British Museum, 3. xxvi+586. London,

1887. Report on the deep-sea fishes collected by H. M. S. Challenger during the years 1873–76. in Report of the voyage of H. M. S. Challenger during the years 1873–76. Zoology, 22. lxv+268, pls. 66. London.

HARRY, Robert R.

1948. The gobies of the Indo-Malayan eleotrid genus *Bunaka*. California Zoological Club, Proceedings 1 (3): 13–18, figs. 6.

HERRE, Albert W. C. T.

1927. Gobies of the Philippines and the China Sea. Bureau of Science, Manila, Monographs 23: 1–352, figs. 6, frontisp., pls. 30.

1933a. On the genera *Ctenogobius* and *Rhinogobius* GILL, *Tukugobius* HERRE, and *Drombus* JORDAN and SEALE. Science 78 (2021): 265.

1933b. A check list of fishes from Dumaguete, Oriental Negros, P. I., and its immediate vicinity. Pan-Pacific Research Institution, Journal 8 (4): 6–11.

1934. The fishes of the HERRE 1931 Philippine Expedition with descriptions of 17 new species. Notes on fishes in the Zoological Museum of Stanford University, 1. 106. Hongkong.

1936. Notes on fishes in the Zoological Museum of Stanford University. IV. New or rare Philippine fishes from the HERRE 1933 Philippine Expedition. Philippine Journal of Science (1936) 59 (3): 357–373, pls. 2.

1941. A list of the fishes known from the Andaman Islands. Indian Museum, Memoirs 13 (3): 331–403.

1945. Two new genera and four new gobies from the Philippines and India. Copeia 1945 (1): 1–6.

1953: Check list of Philippine fishes. Fish and Wildlife Service, U. S. Department of the Interior, Research Report 20: 1–977.

1954. The eleotrid gobies of the Philippines and adjacent regions. Philippine Journal of Science (1953) 82 (4): 345–373.

HUBBS, Carl L. and Mott D. CANNON

1935. The darters of the genera *Hololepis* and *Villora*. Museum of Zoology, University of Michigan, Miscellaneous Publications 30: 1–93, pls. 3.

ILJIN, B. S.

*1927. Table déterminative des Gobiidés des Mers Noires et d'Azow. Information préliminaire. Travaux de l'expédition pour l'exploiration des Mers Noirs et d'Azow 2: 128–143. (in Russian)

*1930. Le système de Gobiidés. Instituto Español de Oceanografía, Trabajos 2: 1–63, figs. 48.

JORDAN, David S. and Carl L. HUBBS

1925. Record of fishes obtained by David Starr JORDAN in Japan, 1922. Carnegie Museum, Memoirs 10 (2): 93–346, fig. 1, pls. 12.

JORDAN, D. S. and John O. SNYDER

1901. A review of the gobioid fishes of Japan, with descriptions of twenty-one new species. United States National Museum, Proceedings 24 (1244): 33–132, figs. 33.

KAROLI, Kános

*1882. Prodromus piscium Asiae orientalis a Domino Joanne XANTUS annis 1868–70 colectorum. Természetraji Füzetek 5: 147–187.

KOUMANS, Frederik P.

1931. A preliminary revision of the genera of the gobioid fishes with united ventral fins. Proefschrift... ix+174. Lisse.

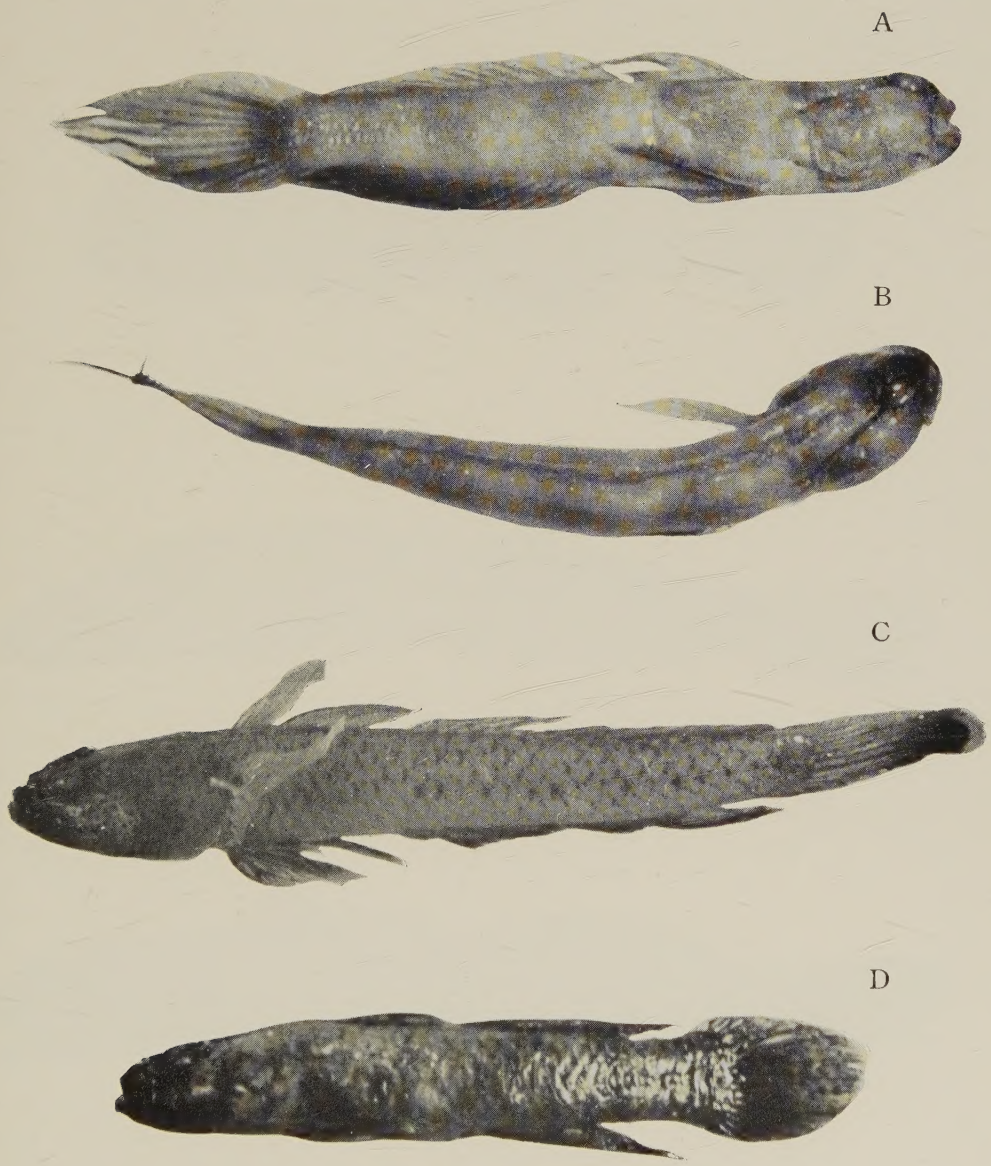
1941. Gobioid fishes of India. Indian Museum, Memoirs 23 (3): 205–329.

1953a. Biological results of the Snellius Expedition. XVI. The pisces and leptocardii of the Snellius Expedition. Temminckia 9: 177–275.

1953b. Gobioidea. in The fishes of the Indo-Australian Archipelago, X. xiii+423, figs. 95, Leiden.

LAMBERTENGHI, Ada
*1919. Distribuzione delle papille cutanee in *Gobius fluviatilis* BONELLI in rapporto a quella dei Gobi marini. R. Comitato Talassografico Italiano, Memoria 70.
LE DANOIS, Yseult
1956. A propos du système muco-sensoriel de poissons orbiculates. Académie des Sciences, Paris, Comptes Rendus des Séances 242 (5): 684–686.
LEKANDER, Bertil
1949. The sensory line system and the canal bones in the head of some Ostariophysi. Acta Zoologica 30: 1–131, figs. 1–67.
MATSUBARA, Kiyomatsu
1955. Fish morphology and hierarchy, 2. v+791–1605, figs. 247, frontisp. Tokyo. (in Japanese)
MATSUBABA, K. and Tamotsu IWAI
1954. Some remarks on the family Liparidae, with descriptions of three new species and two interesting ones of the genus *Liparis*. Faculty of Fisheries, Prefectural University of Mie, Report 1 (3): 425–441, figs. 11.
MORTARA, Silvia
*1918. La disposizione degli organi ciatiformi del genere *Aphya* e suoi rapporti con quella del genere *Gobius*. R. Comitato Talassografico Italiano, Memoria 65.
OCHIAI, Akira, Chûichi ARAGA and Matsuo NAKAJIMA
1955. A revision of the dragonets referable to the genue *Callionymus* found in the waters of Japan. Seto Marine Biological Laboratory, Publication 5 (1): 95–132, figs. 18.
RAFFAELE, Federico
*1889. Nota intorno alle specie mediterranee del genere *Scopelus*. Zoologischen Station zu Neapel, Mittheilungen 9: 179–186, pl. 1.
SANZO, Luigi
1911. Distribuzione delle papille cutanee (organi ciatiformi) e suo valore sistematico nei Gobi. ibid. 20: 251–328, figs. 15, pls. 4.
STARKS, Edwin C.
1930. The primary shoulder girdle of the bony fishes. Stanford University Publications, Univ. Ser., Biol. Scis. 6 (2): 1–93, figs. 38.
STENSIÖ, Erik A:Son
1947. The sensory lines and dermal bones of the cheek in fishes and amphibians. Kungl. Svenska Vetenskapsakademiens Handlinger [3] 24 (3): 1–195, figs. 38.
TAKAGI, Kazunori
1950. On the glossohyal bone of the gobioid fishes of Japan, with some phylogenetic considerations. Japanese Journal of Ichthyology 1 (1): 37–52, figs. 3. (in Japanese with English résumé)
TARANEC (TARANETZ), A.
1934. A short review of the fishes of the genus *Gymnogobius* with a description of one new species and notes on some related. Académie des Sciences de l'URSS, Comptes Rundus 3 (5): 397–400. (in Russian with English résumé)
TOMIYAMA, Itirô
1936. Gobiidae of Japan. Japanese Journal of Zoology 7 (1): 37–112, figs. 44.
WEBER, Max
1913. Die Fische der Siboga-Expedition. in Siboga-Expeditie, 57. viii+710, figs. 123, pls. 12. Leiden.
WEBER, M. and Lieven F. DE BEAUFORT
1911. Index of the ichthyological papers of P. Bleeker. in The fishes of the Indo-Australian Archipelago, 1. xi+410. Leiden.

PL. V.

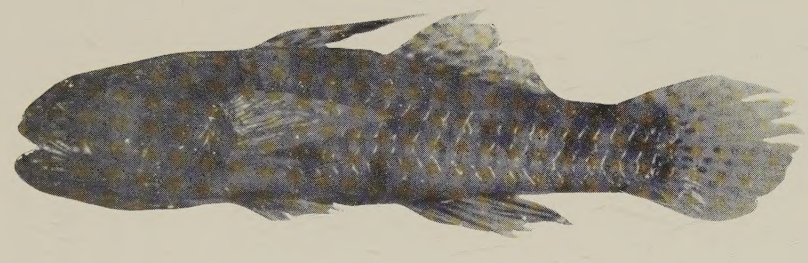


A, B. *Amblyeleotois japonicus*, sp. nov., 84.8 mm. long, female, in lateral (A) and dorsal (B) views.

C. *Ctenogobius dotui*, sp. nov., 28.2 mm. long, male.

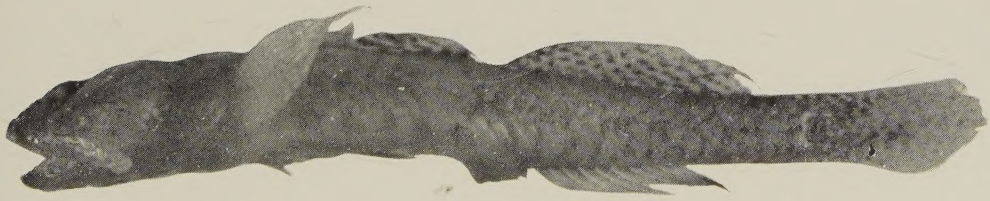
D. *Callogobius sunkan*, sp. nov., type, 59.0 mm. long, male.

E



F

G



E. *Stigmatogobius minutus*, sp. nov., type, 26.2 mm. long, male.

F. *Paleatogobius uchidai*, gen. et sp. nov., 30.0 mm. long, female.

G. *Chaenogobius scrobiculatus*, sp. nov., type, 35.2 mm. long, male.